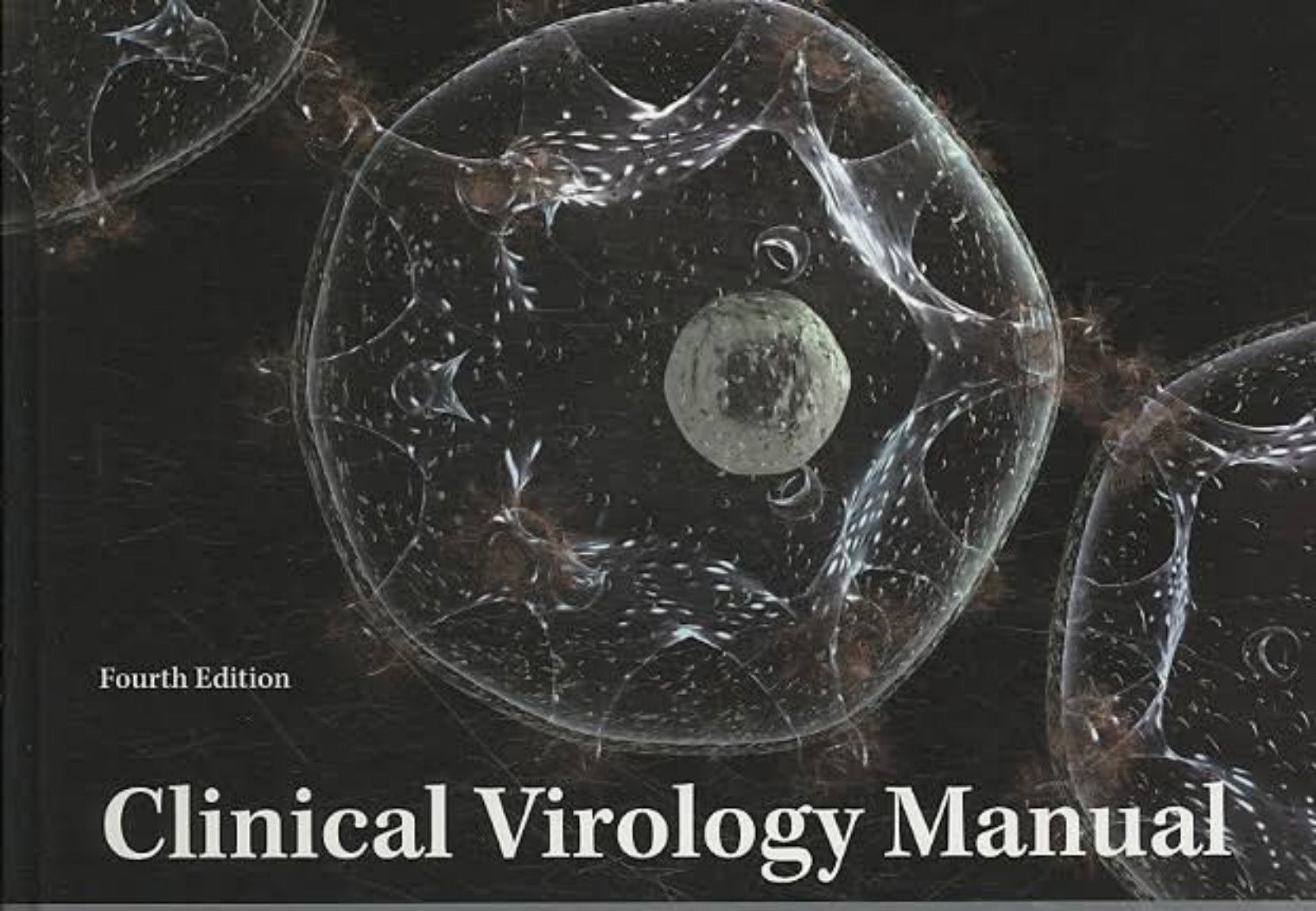

Fourth Edition

Clinical Virology Manual

Editors
Steven Specter
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DEDICATION

We dedicate this edition of the *Clinical Virology Manual* to the memory of our colleague, mentor, and friend, Herman Friedman, who passed away during the summer of 2007. Dr. Friedman's vision was responsible for the initiation of the first edition, and his foresight and insights stimulated the dissemination of information through this virology manual and the Clinical Virology Symposium for a field that continues to expand.

and

To our wives, Randie, Kitty, and Linda

and

Our children, Ross, Rachel, Ryan, Tyler, Brett, Jesse, and Eileen,
whose patience and support sustain us through all our endeavors

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Preface to the Fourth Edition

The aims of this fourth edition of the *Clinical Virology Manual* remain the same as the those for the first edition; thus, the original preface is included to describe those goals. Updated from the third edition, this new edition includes 34 chapters and 2 appendices. The listing of laboratories offering viral services has been deleted from the original section on Reference Laboratories in the appendix because there are now many more virology services and the list changes too frequently. The reader is referred to his/her state health laboratories for assistance as needed. Many of the chapters in this edition have been updated and expanded, while chapters on some of the less used virology techniques of the past have been deleted, including chapters on the interference assay, radioimmunoassay, complement fixation, immune adherence hemagglutination, and automation. This edition includes separate chapters describing papillomaviruses and polyomaviruses, which were previously dealt with jointly as papovaviruses. The chapter updates are intended to address the modernization that has occurred in the past several years, with a strong emphasis on molecular diagnostics. In the Viral Pathogens section we have included information on several newly described viruses including human metapneumovirus, West Nile virus, bocaviruses, newer influenza and adenoviruses, and others. The information on the federal laboratory organization at the Centers for Disease Control and Prevention (CDC) and state public health laboratories in the Reference Laboratories section has also been updated.

This edition brings one other major change, the inclusion of a new editor. We are pleased that the American Society for Microbiology is continuing as our publisher for this edition and hope that ASM members as well as nonmembers will find this manual a useful adjunct to the *Manual for Clinical Microbiology* and *Manual for Molecular and Clinical Laboratory Immunology*. There are a number of chapters for which the authors have changed as a result of change of professional focus or retirement. We thank all of those authors for their efforts. We hope that this edition is a credit to those who preceded this effort, especially Jerry Lancz, who helped to start this series.

STEVEN SPECTER
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Preface to the First Edition

Clinical virology is an area that is undergoing rapid expansion. As a service for patient care, the utility of the clinical virology laboratory has increased significantly in the past decade. Due to the availability of commercial test kits, sophisticated yet simple diagnostic reagents, and the standardization of laboratory assays, accurate, reliable and, in many instances, rapid protocols are currently available for the diagnosis of a variety of viral agents producing human infections. Thus, the demands (on both the physician and the clinical laboratory virologist) for the diagnosis of viral infections will continue to increase. With this in mind, this volume is written as both an aid to the clinician and as a guide for the clinical laboratory.

This manual has three sections. The first describes laboratory procedures to detect viruses. The individual chapters deal with quality control in the laboratory and specimen handling, areas that are critical for an effective diagnostic laboratory. This is followed by individual chapters that provide information or a detailed protocol on how to set up and test samples for viral diagnosis using this technique. Both classical and the newer, more experimental techniques are described in detail.

The second section focuses on the viral agents. Viruses are grouped into chapters based on a target organ-system categorization. In this way, viruses producing infection in a particular organ or tissue are discussed and compared in a single chapter. This approach more accurately reflects the problems and choices faced by the attending physician and clinical technician for the diagnosis of a viral infection. Each chapter includes information relating basic, pathogenic, immunologic, and protective measures concerning each virus group, as well as information on its isolation, propagation, and diagnosis. This section also includes a chapter on *Chlamydia*. There are two reasons for including this family: the clinical laboratory often isolates and diagnoses *Chlamydia*, and the techniques used in its isolation and diagnosis are used in other instances.

The third section is designed to be used for reference. Here we supply information about Federal Reference Laboratories at the Centers for Disease Control and their role in the diagnosis of viral infection. The diagnostic and regulatory activities of state health laboratories and services available at individual hospital laboratories are provided in survey form. This listing is somewhat incomplete in that it contains information provided in response to an initial questionnaire and follow-up.

The aim and scope of this volume are service: to the physician, as a source of basic and clinical information regarding viruses and viral diseases, and to the laboratories, as a reference source to aid in the diagnosis of virus infection by providing detailed information on individual techniques and the impetus to expand services offered.

STEVEN SPECTER
GERALD LANCZ

LABORATORY PROCEDURES FOR DETECTING VIRUSES

I

Quality Assurance in Clinical Virology

CHRISTINE C. GINOCCHIO

1

Today, medical practitioners rely on the ability of the clinical laboratory to provide key scientific data used for the diagnosis, treatment, and monitoring of persons with viral diseases. Therefore, the accuracy of test results is critical, and ongoing quality assurance (QA) and quality improvement programs are key factors in maintaining service excellence. All members and levels of the laboratory staff, from lab assistants to directors, are responsible for QA and improvement and must play an active role on a daily basis. QA programs must be comprehensive so that all aspects of the laboratory testing are monitored, including the preanalytical, analytical, and postanalytical phases. Another key component of QA is maintaining staff proficiency and competency. Quality improvement programs are necessary to provide a surveillance mechanism that will identify specific phases of the testing that are suboptimal and need improvement. In Nutting's 1996 report of a prospective study of the type and frequency of laboratory testing problems in primary care physicians' offices during a 6-month period, a rate of 1.1 problems per 1,000 visits was found (Nutting et al., 1996; Warford, 2000). Twenty-seven percent of these test problems had an impact on patient care, including serious effects such as unnecessary hospitalization, prolonged hospital stay, more-invasive diagnostic procedures, and delays in treatment. However, only 25% of the laboratory problems involved test analysis or inconsistent results; 75% of errors occurred in specimen collection and transport (43%) or timely provider notification of results (32%). This and other studies (Boone et al., 1982; Bartlett et al., 1994) confirm the need for laboratory involvement in improving the total testing process if laboratory services are to be meaningful and beneficial in patient health care (Warford, 2000).

Finally, the laboratory must establish and comply with written policies and procedures that maintain an ongoing process to monitor, assess, and when indicated, correct problems identified in all phases of testing. The assessment must include documentation of problems, communication with appropriate persons, a review of the effectiveness of corrective actions taken to resolve problems, revision of policies and procedures necessary to prevent recurrence of problems, and discussion of assessment reviews with appropriate laboratory and clinical staff (Health Care Financing Administration [HCFA], 1992; National Committee for Clinical Laboratory Standards [NCCLS] 1998a, 2004a; Joint Commission on Accreditation of Healthcare Organizations [JCAHO], 1998).

REGULATORY REQUIREMENTS

Effective September 1992, with the implementation of the federal Clinical Laboratory Improvement Amendments (CLIA) of 1988 (CLIA-88), all laboratory testing performed on humans (except research) in the United States is regulated by the Centers for Medicare and Medicaid Services (CMS), formerly known as the HCFA, unless the state health department regulations exceed and are approved by CMS (Centers for Disease Control and Prevention [CDC], 1992; HCFA, 1992). In total, CLIA covers approximately 198,000 laboratory entities. The Division of Laboratory Services, within the Survey and Certification Group under the Center for Medicaid and State Operations, has the responsibility for implementing the CLIA program (HCFA, 1992). The CLIA program is designed to ensure quality laboratory testing, and all clinical laboratories must be properly certified to receive Medicare or Medicaid payments.

The provisions of CLIA-88 include licensure, inspections conducted by CMS or approved organizations, such as the College of American Pathologists (CAP) and JCAHO, and sanctions for failure to meet mandated standards (Warford, 2000). The stated purpose of CLIA-88 regulation of laboratories is to improve laboratory quality and achieve accurate and reliable laboratory results. The main quality standards of the regulatory and accrediting organizations can be categorized as (i) personnel qualifications, responsibilities, and competency assessment; (ii) proficiency testing for all analytes and staff; (iii) written and approved procedures; (iv) method verification and validation; (v) test reagent and equipment quality control (QC) and preventive maintenance; and (vi) patient test management. The process must include ongoing assessment, and the goal is to provide for improvement of all laboratory services. Please refer to references listed that provide in-depth information regarding U.S. clinical laboratory regulations and accreditation requirements as well as useful QA resources.

STAFF REQUIREMENTS, TRAINING, AND COMPETENCY

Quality viral diagnostic services are highly dependent on well-trained and competent laboratory personnel (Bartlett et al., 1994; JCAHO, 1998; NCCLS, 1998a, 2004a, 2004c; Sharp, 2003; Sewell, 2005). The laboratory director is responsible for providing written qualifications, duties, and responsibilities

for all staff positions in accordance with local, state, and federal requirements and ensuring that staffing levels are adequate for the type and volume of testing performed. Excessive workloads are not consistent with quality, particularly with subjective tasks requiring judgment, such as microscopy (Warford, 2000). Staff qualifications for education, experience, training, and licensure or certification vary greatly among the regulatory and accrediting agencies, with CLIA-88 having the minimum requirements (HCFA, 1992; Sewell, 2005). Several states require specific licensure for both laboratory directors and technical staff.

CLIA-88 categorized virology testing as moderate- and high-complexity testing, with only a few infectious mononucleosis serology kits and rapid antigen immunoassays (e.g., for influenza virus and respiratory syncytial virus [RSV]) listed as "waived" tests, i.e., exempt from many CLIA-88 regulations. Virology laboratories can offer level-one testing, which consists of immunoassays for antigen detection without microscopy, or level-two high-complexity testing for virus isolation and identification and all other viral diagnostics. Because most virology methods are complex and subjective, requiring independent analysis and decisions, adequate education and training in theory and methods are essential for quality results. Several studies have correlated the level of education, training, and certification or licensure with laboratory performance quality, as measured in proficiency surveys (Gerber et al., 1991; Woods and Bryan, 1994; CDC, 1996; Shahangian, 1998; Warford, 2000; NCCLS, 2004c; CLSI, 2007a).

Training verification and ongoing competency of laboratory staff is mandated by CLIA-88 and is another of the main CMS inspection deficiencies cited (Table 1). The laboratory should have a comprehensive training manual and documentation that all personnel have read and understand the preanalytical, analytical, and postanalytical test requirements, have been trained to perform the procedure, are proficient in the testing, and are able to report patient results accurately (Bartlett et al., 1994; JCAHO, 1998; NCCLS, 1998a, 2004a, 2004c; Sharp, 2003; Sewell, 2005). Training verification and competency assessment can be documented (Table 2) by observation, use of training checklists created from the major and critical steps of the procedure manual, written tests, and requiring the staff to test and pass a blinded proficiency panel prior to reporting clinical results. Training can be provided by other trained laboratory staff or by test vendors at their facilities or onsite. The manual should also address training and competency with the laboratory information systems, reporting policies, and institutional mandatory topics, such as Health Information Portability and Accountability Act requirements, fire and safety, administrative policies, emergency management, corporate compliance, and infection control.

Once laboratory staff have been initially trained, it is essential to ensure ongoing competency and education, not only with current testing procedures but also with the rapidly changing field of virology in general. There are several methods that can be used to provide continuing education, including in-house lectures, teleconferences, educational internet programs or CDs, and attendance at local, state, or national workshops or scientific conferences. Competency assessment is a daily process and should be formally documented at least yearly for each staff member. Methods to assess individual competency (Table 2) must include (i) direct observation of test performance, instrument maintenance and function checks, microscopy, and interpretation; (ii) monitoring worksheets, result recording, and reporting; (iii) testing of proficiency samples, previously analyzed samples, and blind controls or reference samples; and (iv) daily review of QC and preventive maintenance records (Warford, 2000). Competency can also be evaluated by monitoring (i) failed test runs and QC failures; (ii) numbers of corrected reports due to technical or clerical errors; (iii) correlation of preliminary results with final or repeat results for a pattern of inconsistencies; and (iv) client and staff complaints. Finally, technical staff should be evaluated for the ability to recognize unusual results and solve problems when test failures occur. Deficiencies must be documented, a corrective action plan followed, and the outcome assessed. The identification of common deficiencies would indicate that the laboratory's processes for recruiting, staffing, training, continuing education, and retention of a qualified staff need to be critically reviewed and corrected appropriately (Warford, 2000).

TABLE 1 Top CMS inspection CLIA-88 deficiencies cited, 1996 to 1998

PT program for each specialty and subspecialty inadequate
QA plan; lack of comprehensive written plan for maintaining quality of overall testing process, identifying problems, and implementing corrective action
QC not documented with at least two levels of controls for each day of testing
Preventive maintenance and function checks of instrumentation inadequate
Competency assessment program of staff performance inadequate
Daily supervisory review of QC, PM, and patient test results not performed
Procedure manual and job descriptions without lab director's written designation of responsibilities and duties of staff
Correlation of multiple tests methods for same analytes not documented

[a]Sources: Chapin and Baron, 1996; Belanger, 1998.

TABLE 2 Staff training verification and competency assessment documentation[a]

Technical supervisor must assess and verify staff performance of procedures at least annually by use of the following:
• Direct observation of routine test performance, instrument maintenance and function checks, and microscopy and interpretation
• Monitoring worksheets, result recording, and reporting
• Testing proficiency samples, previously analyzed specimens, blind controls, and/or reference samples
• Daily review of QC records and preventive maintenance records
• Monitoring of failed test runs, unacceptable QC results, and run contamination
• Correlation of preliminary results with final or repeat results for patterns of inconsistencies
• Additional procedures such as written or verbal tests, continuing education, problem solving of test failures, evaluation of critical incidents, error reports, or client and staff complaints
• Reevaluation required with each change in methods

[a]Source: Warford, 2000.

PROCEDURE MANUAL

A clear and concisely written procedure manual for all tests performed by the laboratory must be available at the bench and followed by the laboratory personnel. The procedures should be written according to the guidelines established by the CLSI (CLSI, 2006b), formerly known as the NCCLS. Manuals should also address specific safety issues, such as the use of biohazard hoods, protective personnel equipment, transport and shipping of biological specimens or infectious agents, agents of bioterrorism, disposal of infectious waste, and emergency management. Textbooks, package inserts, and manufacturer's operator manuals may be used as supplemental references but do not replace the laboratory's written test procedures.

The required components of the laboratory procedure manual must address the preanalytical, analytical, and postanalytical phases of testing and are listed in Table 3 (HCFA, 1992; CLSI, 2006b). Instructions that assist medical personnel in appropriate test selection and ordering, patient preparation, sample collection, storage, and transport must be available either electronically or as laboratory testing manuals for distribution to all clients, including outreach physicians' offices, long-term care facilities, clinics, or hospital nursing units.

In addition to manuals that contain specific test protocols, the virology laboratory must have written protocols that describe the QA and quality improvement programs. The policies should include (i) requirements for assessment of the preanalytical, analytical, and postanalytical phases of testing; (ii) ongoing verification requirements for proficiency testing; (iii) safety; (iv) technical training; and (v) ongoing competency assessment. Yearly, the laboratory should select and monitor specific quality indicators and document outcomes and corrective actions.

All procedures should be maintained under a document control system according to the International Organization for Standardization (ISO) 3500 regulations. This includes records of date of initial use, dates of procedural changes, and date of discontinuance. All new procedures and changes in procedures must be approved, signed, and dated by the current laboratory director before use. Discontinued procedures must be maintained by the laboratory for 2 years. There must also be documentation that all technical staff who perform the procedures have read the procedure and any supplemental modifications. All procedures must be reviewed yearly by the laboratory director.

TABLE 3 Mandatory components of procedure manuals[a]

Requirements for patient preparation
Specimen collection, labeling, storage, preservation, transportation, processing, and test referral
Criteria for specimen acceptability and rejection
Background and significance of the test
Explanation of the test methodology
Detailed step-by-step performance of the procedure, including test calculations and interpretation of results
Preparation and storage of slides, solutions, calibrators, controls, reagents, stains, and other materials used in testing
Calibration and calibration verification procedures
Established and verified reportable range for test results for the test system
Control procedures and corrective action to take when calibration or control results fail to meet the laboratory's criteria for acceptability
Limitations in the test methodology, including interfering substances
Reference intervals (normal values)
Reporting formats
Critical or alert values
Pertinent literature references
Alternative method if test or system is inoperative

[a]Sources: HCFA, 1992; CLSI, 2006b.

PROFICIENCY TESTING

CLIA-88 has adopted external, graded proficiency testing (PT) programs as the main indicator of the quality of laboratory testing performance (HCFA, 1992). In addition, PT can serve proactively as a quality management tool (Boone et al., 1982; Hoeltge and Duckworth, 1987; CDC, 1992; ISO and International Electrotechnical Commission [IEC], 1997; JCAHO, 1998; Shahangian, 1998; CLSI, 2005c, 2007a). All laboratories must participate in PT programs for each analyte or test for which patient testing is performed. CLIA-approved 2007 PT programs and analytes offered are listed in Table 4. CLIA-approved PT programs must include samples for viral antigen detection (rapid antigen tests for influenza viruses A and B, RSV, and rotavirus; antigen detection by immunofluorescence for influenza viruses, parainfluenza viruses, RSV, adenovirus, herpes simplex virus, varicella-zoster virus, and cytomegalovirus) and virus isolation and identification. The PT program must include the more commonly identified viruses, and the specific organisms found in the PT samples may vary from year to year. The PT program must provide a minimum of five samples per testing event, with a minimum of three testing events at approximately equal intervals per year. Guidelines for PT by laboratory comparisons also have been established by the ISO and IEC (ISO and IEC, 1997). Information in the guidelines addresses the development and operation of PT schemes and the selection and use of PT schemes by laboratory accreditation bodies.

For any analyte that is not evaluated or scored by a CMS-approved PT program, the Clinical Laboratory Improvement Advisory Committee recommends that the laboratory

TABLE 4 CLIA-approved PT programs as of August 2007

Name and location	Analytes offered
American Academy of Family Physicians, Leawood, KS	Direct viral antigen detection
American Association of Bioanalysts, Brownsville, TX	Direct viral antigen detection
American Proficiency Testing Programs, Traverse City, MI	Direct viral antigen detection
CAP, Northfield, IL	Direct viral antigen detection, viral isolation and identification, molecular detection
Medical Laboratory Evaluation, Washington, DC	Direct viral antigen detection
Wisconsin State Laboratory of Hygiene, Madison, WI	Direct viral antigen detection
New York State Department of Health, Albany, NY	Direct viral antigen detection, virus isolation and identification

develops an in-house PT program to ensure that the laboratory tests five sample challenges, preferably three times per year, to verify the accuracy of the test or procedure it performs (HCFA, 1992; NCCLS, 2002; CLSI, 2005c). The testing of in-house PT samples must comply with the same testing guidelines as those required for CLIA-approved PT programs. Sources for in-house PT materials can include blinded commercial panels of known reactivity, samples split with a reference laboratory, or previously tested samples of known reactivity. Several studies have demonstrated that the best measurement of laboratory routine performance can be accomplished with samples split and relabeled prior to receipt in the laboratory (Boone et al., 1982; Farrington et al., 1995; Gray et al., 1995a, 1995b; Yen-Lieberman et al., 1996; Shahagian, 1998). The CDC also offers test panels for both standard and rapid human immunodeficiency virus antibody testing, twice yearly, as part of its Model Performance Evaluation Program. Although they are not graded challenges, results are provided with comparisons to those obtained by other participating institutions.

Written policies defining the process for PT must be clearly defined and understood by all personnel. PT must be incorporated into the routine daily laboratory testing and performed in the same manner and with the same staff as routine patient samples. PT challenges should be rotated among shifts and personnel who perform the testing. The laboratory must identify viruses to the same extent it performs these procedures on patient specimens. Supervisory personnel should ensure that their staff do not perform "extra" testing (testing not normally performed for routine clinical samples or duplicate testing) to confirm that their initial results were correct. During the testing period, interlaboratory communication, comparison of results, and referral of the sample to a reference laboratory for identification or confirmation is strictly prohibited. All submitted result forms and final grading reports should be reviewed and signed by the testing personnel, laboratory supervisor, and laboratory director.

The accuracy of a laboratory's response for a CLIA-approved PT challenge is determined by comparison of the laboratory's response for each sample with the response that reflects agreement of either 80% of 10 or more referee laboratories or 80% or more of all participating laboratories (HCFA, 1992; CAP, 2007). Unsatisfactory scores can also result from a failure to participate in a testing event or failure to return PT results to the PT program within the time frame specified by the program. However, consideration may be given to laboratories if the PT program was notified as to the circumstances of the failure. Failure to attain an overall testing event score of at least 80% is unsatisfactory performance, and laboratories that fail consecutive challenges or two of the three annual testing events are subject to severe sanctions. For in-house-developed PT challenges, similar guidelines should be developed for grading of results. For an unsatisfactory testing event, the laboratory must review its policies for PT, provide appropriate training, and employ the technical assistance necessary to correct problems associated with the PT failure. Any type of PT assessment is useless without investigation and efforts to improve system problems. The investigative process and documentation of any remedial action must include and be completed by all persons involved in the PT failure, including the laboratory director. Documentation must be maintained by the laboratory for 2 years from the date of participation in the PT event.

Inadequate PT performance is the most common post-CLIA-88 inspection citation (Table 1) (Chapin and Baron, 1996; Belanger, 1998; Warford, 2000). PT failures provide an opportunity for evaluation of factors contributing to test performance problems (Table 5) (Warford, 2000), and use of total quality management methods with staff input from all sections and levels is recommended and outlined by CLSI and others (Engebretson and Cembrowski, 1992). Investigations by CDC and CAP showed that approximately 20% of repeated PT failures have no cause identified by the laboratory and that on-site technical consultation was required for performance improvement (Boone et al., 1982; Hoeltge and Duckworth, 1987).

Unfortunately, known PT samples are an imperfect measure of a laboratory's performance accuracy and reliability because they (i) are recognized challenges which have penalties for failure and are prone to special attention; (ii) test only the analytical phase of testing, not specimen collection, transport, or usual result reporting; (iii) consist of laboratory-adapted virus(es) or pooled, processed body fluids spiked with an analyte which may have a matrix effect, which renders them inaccurate with certain methods; and (iv) cannot test analyte concentrations near the assay cutoff due to nonconsensus results with borderline levels (Warford, 2000). However, PT samples do still detect staff human errors, serve as a form of competency testing, and identify technical problems and some poorly performing methods. Residual PT samples are excellent resource materials for new test validations and technical training. Proficiency sample testing and the analysis of results provided by programs such as CAP PT surveys also provide an educational resource for the laboratory (Warford, 2000).

TABLE 5 Troubleshooting unacceptable patient or PT results[a]

Procedure or method
• Equipment, reagents, standards, QC materials
• Limitations of methodology—sensitivity, specificity, precision, linear range
• Written procedure erroneous
Technical factors
• Incubation time, temperature, humidity, carbon dioxide
• Pipetting, dilutions, calculations
• Misinterpretation, not following written protocol
Staff or staffing
• Training, experience, continuing education
• Use of overtime, per diem, rotating staff
• Workload-to-staff ratio
Clerical error(s)
• Mislabeling, transcription, units, computer entry
Sample or sampling
• Transport time and/or temperature
• Interfering substances, contamination
• Organism or analyte not present or not viable on receipt
Obtain input on preventive measures from lab staff and others

[a]Source: Warford, 2000.

QA

Preanalytical Phase

QA for the preanalytical phase of testing begins with laboratory compliance, with the regulation that an appropriate electronic or paper test requisition form is submitted from an authorized person and, if a verbal request is made, that the appropriate authorization is received within 30 days (HCFA, 1992). The components of the requisition need to be compliant with the general CLIA laboratory requirements; however, for virology specimens, it is especially essential to have an accurate and specific sample type listed, indication of suspected virus(es), and any clinical information (e.g., immune suppression) that may influence the selection of the tissue culture medium. The date and time of specimen collection are critical for assessing the quality of the specimen and the ability to recover viruses, as a delay in delivery to the laboratory can significantly reduce the recovery of many labile viruses, such as RSV. Entry of the test requisition information into a record system or a laboratory information system can be a source of error, so the laboratory must ensure the information is transcribed or entered accurately. This can be monitored by performing a second-pass inspection of each requisition. Alternatively, although most random errors will not be detected, consistent errors may be detected by routinely selecting random requisitions and verifying that the information (e.g., patient identification, medical record number, date of birth, tests ordered, specimen source) entered into the patient's sample record and the tests ordered were accurate.

The laboratory must provide to clients written policies and procedures for patient preparation, specimen collection, specimen labeling (including patient name or unique patient identifier), specimen storage and preservation, conditions for specimen transportation, specimen processing, specimen acceptability, and rejection. Clients must adhere to all of these policies. This is to ensure that the specimen is collected properly and is appropriate for the targeted virus(es) and that the integrity of the specimen is maintained. Specialized instructions are also essential, considering that at any time the laboratory could receive specimens that may contain highly infectious agents, such as avian influenza virus or the severe acute respiratory syndrome (SARS) coronavirus, viruses that should not be cultured in routine clinical laboratories. If referral is required, laboratories should follow any local, state, or federal regulations and can only refer testing to a CLIA-certified laboratory or a laboratory meeting equivalent requirements, as determined by CMS (HCFA, 1992; NCCLS, 1998b).

Specimens that are inappropriately collected, stored, or preserved are one of the major sources of poor quality results in the virology laboratory (Lennette, 1995; CLSI, 2005a). Labile viruses die quickly, thus reducing virus titers and recovery. Nucleic acids can quickly degrade, resulting in false-negative results with molecular-based assays. The laboratory should reject these samples and request a new specimen. Examples of common specimen rejection criteria are listed in Table 6. If it is not possible to collect a new sample, the laboratory must assess the integrity of the sample and determine if testing could still be performed, and a notation should be included along with the test results indicating the potential impact on the test results. This type of information is important for monitoring and assessing the laboratory's procedures for sample collection, transport, and test performance. For example, if a significant discordance is noted with poor virus recovery in culture and better detection by testing that does not require live virus, such as direct immunofluorescence (direct fluorescent-antibody assay [DFA]) or rapid membrane enzyme immunoassay (MEIA), an evaluation of site compliance with appropriate specimen collection, storage, and transport protocols would be indicated. These data may also guide the selection of the most appropriate test methods for particular viruses and lead to an improvement in specimen collection tools and methods.

Analytical Phase

Verification and Validation

Verification and/or validation studies of new tests, kits, or reagents are essential prior to clinical use and the reporting of patient results (Elder et al., 1997; Association for Molecular Pathology [AMP], 1999; NCCLS, 2003, 2004b; CLSI, 2005b, 2006c, 2006e, 2007b). Validation studies, performed by the test manufacturer (U.S. Food and Drug Administration [FDA]-cleared assays) or by the laboratory (kits labeled research use only [RUO] or investigational use only [IUO] or as analyte-specific-reagent [ASR]-developed assays) determine the performance characteristics (i.e., precision, accuracy, sensitivity, specificity, reportable range, etc.) of the test. Verification is defined as the ongoing process that confirms the specified performance characteristics of the assay, as previously determined by the manufacturer or laboratory during the validation studies. For FDA-cleared instruments, kits, and test systems (both nonmolecular and molecular based), the laboratory must verify the manufacturer's performance claims for accuracy, precision, and reportable range, as stated in the package insert. The laboratory must also verify that the manufacturer's reference intervals (normal values) are appropriate for the laboratory's patient population. Verification usually consists of parallel testing the new product with a standard method of known performance characteristics. For qualitative assays, a minimum of 20 known positive clinical samples and 50 negative clinical samples have been recommended for this evaluation by McCurdy and colleagues (Elder et al., 1997). Negative samples should include those containing other nontarget viruses commonly isolated from the same source (e.g., other respiratory viruses) or genetically similar viruses that may cause a false-positive result due to cross reactivity (e.g., enteroviruses and rhinoviruses). For quantitative assays, in addition to clinical samples of known reactivity, the laboratory must also verify the lower and upper limits of detection, linearity across the dynamic range of the assay, reproducibility, and precision of the assay. Verification must include all FDA-approved sample types (or matrices) that will be used for testing with the assay (HCFA, 1992; NCCLS, 2003). The use of alternative sample types, not FDA cleared, requires more extensive validation studies, as described below for non-FDA-cleared tests. Clinical samples are essential for accurate verification studies. However, a sufficient quantity of positive or negative clinical material may not always be available to the laboratory. Alternative sources can include split samples sent to a reference laboratory, interlaboratory exchange of samples, proficiency materials, and control material spiked into appropriate matrices at clinically relevant concentrations. When possible, verification studies should be performed blinded to comparator results across multiple days, multiple runs, or batches and using several technical personnel. Verification testing must be performed in the same manner as patient samples, following the manufacturer's kit instructions, and include

TABLE 6 Examples of specimen rejection criteria[a]

Problem	Specimen	Test	Action	
			Reject (phone for new sample)	Process and test with disclaimer
Delay in transit	Clotted blood	Serology		>24 h
	Whole blood (unspun)	Culture/PCR	>12 h (whole blood)	6–12 h (whole blood)
	Serum or plasma (RT)	PCR	>72 h (RT)	25–72 h (RT)
	Serum or plasma (cold)	PCR		>72 h (cold packs, refrigerated)
	PPT tube (unspun)	PCR	>12 h (unspun, whole blood)	6–12 h (unspun, whole blood)
	PPT tube (spun)	PCR	>72 h (RT)	25–72 h (RT)
	PPT tube (spun)	PCR		>72 h (cold packs, refrigerated)
	Stool	*Clostridium difficile* toxin		>4 h C. *difficile* stool
		Viral culture		>48 h (refrigerated) for viral cultures
Heparin (green top)	Whole blood	PCR	Any, cannot use for PCR	
Hemolysis	Serum	Serology	Looks like whole blood	Mild/moderate hemolysis (note serum appearance in computer)
Lipemia/icterus	Serum	Serology		Note appearance in computer
Mislabeled or unidentified	Any (except surgery)	All	Reject and recollect	Tissue/CSF (have physician identify and sign, add disclaimer)
Dry swab, wood, calcium alginate, or charcoal swabs	Swab	Culture		Note unsatisfactory swabs in computer with disclaimer
Container gross external contamination	Any (except surgery)	Any	Reject and recollect	Tissue/CSF (disinfect with bleach)
Duplicate (<24 h)	Any except surgery (BAL fluid, biopsy specimen, CSF)	Any	Reject duplicate blood, urine, stool	Process if requested by physican
Fixative (Formalin)	Any	Any	Reject and recollect	
Non-VTM (Bacti, Culturette)	Swab	Culture/DFA	Cannot use Culturette for DFA/EIA or *Chlamydia*	Can use Culturette for viral culture (transfer to VTM as soon as possible)
Nonstandard source or collection	Sputum or stool for respiratory viruses	Culture/DFA	Reject, recollect NP, BAL fluid, or NP-OP combination	Add disclaimer
QNS	Any	Any		Call for physician's test priority list
Inadequate cellular material	Lesions, swabs	DFA	Call for recollection	

[a]Abbreviations: RT, room temperature; PPT, plasma preparation tube; VTM, viral transport medium (SP buffer); NP, nasopharyngeal; OP, oropharyngeal; BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; QNS, quantity not sufficient. Source: Warford, 2000.

the appropriate controls, as designated by the manufacturer and regulatory agencies. Once studies are completed, the laboratory can provide test results within the laboratory's stated performance specifications for each test system (HCFA, 1992).

The validation of new tests, kits, or reagents labeled for RUO or IUO or as ASRs or in-house-developed assays requires a significantly more extensive validation study, as no manufacturer's claims can be made regarding their use, performance, or interpretation of the data (NCCLS, 2003, 2004b; CLSI, 2005b, 2006a, 2006c, 2007b). In addition, for ASRs and in-house-developed assays, the laboratory must also develop and optimize the actual testing protocol. For non-FDA-approved methods, establishing the performance characteristics of accuracy, precision, reproducibility, analytical sensitivity (limit of detection and/or limit of quantitation), specificity, interfering substances, and reportable and reference ranges is CLIA mandated. The validation process must include all phases of the testing and, for optimal evaluation of the results, be performed across multiple days, multiple runs, or batches and using several technical personnel.

For molecular-based assays, validation studies must determine the most appropriate nucleic acid extraction procedures for all matrices to be tested and assess the ability of the procedure to remove amplification inhibitors and the potential for sample-to-sample cross-contamination (CLSI, 2005b).

In addition, the laboratory must establish the test system's calibration and control procedures based upon the performance specifications verified during the validation process. Once the analytical validation is complete, the laboratory must validate the clinical sensitivity, clinical specificity, and positive and negative predictive values of the test. In certain instances, the laboratory must also establish the clinical utility of the test and provide specific guidelines for the use and interpretation of the test. Recommendations for in-house-developed molecular assay validation are specified by CLSI in several documents (NCCLS, 2003, 2004b; CLSI, 2006a, 2006c, 2007b), the AMP (AMP, 1999), and the American Society for Microbiology *Cumitech 31* (Elder et al., 1997). These authors suggest that home brew microbiology assays should be clinically validated with 50 samples known to contain the microbial target and 100 analyte-negative specimens. For some viruses, particularly those that are difficult to culture, 50 positive patient samples may not be available within the institution and collaboration with a reference or large public health laboratory is recommended in addition to obtaining reference standards from commercial or government sources.

Of particular note is the difficulty in sufficiently validating multiplex molecular assays (CLSI, 2007b). Multiplex assays may (i) require more-stringent conditions for nucleic acid isolation and sample purity, (ii) have to be validated using multiple sample types, (iii) have different requirements for sample input volumes, (iv) have to detect different types of targets (e.g., RNA and DNA), and (v) contain more-complex reagents. Analytical validation studies should test the multiplex system in its final format so that the laboratory can assess target competition, cross-reactivity among the different primers and probes, and potential crossover of signals between analytes that could lead to potential false-positive results. The determination of what constitutes a positive and a negative sample needs to be established for each analyte in the multiplex. Validation studies for multiplex assays must demonstrate equal detection of all potential targets, when present as the sole analyte and also when present in combination with other analytes detected simultaneously by the system (i.e., mixed infections). The availability of a method for comparison and suitable validation materials, including reference and test materials and clinical samples containing the targeted analytes, may be limited. Finally, multiplex assays may also require more-complex software algorithms for interpretation of test results.

A detailed description of the verification or validation studies should be provided, all raw data from test results should be retained, and a summary of the results should be signed by the performing technologist(s), laboratory supervisor, and director. Statistical programs should be used to assess all verification and validation data. In some institutions, validation data from new assays must be approved by a specified committee to ensure that a comprehensive study was performed and that results were within acceptable limits prior to use in patient testing.

QC

QC is an integral part of daily laboratory testing and is intended to ensure the performance of test systems and the accuracy of patient results. QC systems must be designed to detect immediate errors that are the result of test system failure, adverse environmental conditions, and poor operator performance. Therefore, QC materials should be tested along with patient samples and in an identical manner. Reference intervals (normal ranges) must be established based upon studies of patient samples that are appropriate for the patient population served by the laboratory. The laboratory must twice yearly evaluate and define the relationship between test results obtained for the same analyte when using different methodologies, instruments, or multiple testing sites (HCFA, 1992).

All QC procedures and results must be documented appropriately and actively reviewed on a defined regular basis (daily by testing personnel and at least monthly by the laboratory director or designee), and corrective action must be implemented in a timely manner (HCFA, 1992; CAP, 2007). More-stringent review of QC data should be considered for assays that are noted to be problematic. A QC failure is noted when test systems (equipment or methods) do not meet the laboratory's verified or established performance claims. Examples of failures include out-of-range controls or calibrators, instrument failures, and patient results outside the normal range of reporting. If QC performance does not meet specifications, patient results should not be released until such time that the cause of the QC failure has been investigated and corrected and the results deemed acceptable. Review of QC results over time is also important to identify trends or shifts in expected results that may affect the accuracy and precision of test performance resulting from subtle system failures, environmental conditions, and variance in technical personnel performance. This is particularly important for quantitative assays. Commercially available computer- or laboratory information system (LIS)-based QC programs are excellent tools the laboratory can use to track results over time.

Reagents, Kits, and Equipment

Monitoring of equipment and environmental conditions is required at least each day of use for all sections of clinical virology. Common equipment requirements include instrument function tests, including background or baseline checks each day of use, equipment calibration, preventive maintenance, safety checks, and service according to the manufacturer's instructions. The frequency should be determined in accordance with manufacturer's recommendations, regulatory requirements, and extent of equipment use. All function checks must be within the manufacturer's established limits before patient testing is conducted. Equipment and instruments must be protected from fluctuations and interruptions in electrical current that adversely affect patient test results and test reports. The laboratory must establish its own maintenance program for the use of equipment, instruments, or test systems commercially available, modified by the laboratory, or developed in-house or if maintenance and function check protocols are not provided by the manufacturer. The maintenance protocol must ensure that the equipment, instrument, and test system performance are appropriate for accurate and reliable test results and test result reporting. Instrument calibration and calibration verification procedures must be performed and documented to substantiate the continued accuracy of the test system throughout the laboratory's reportable range of test results (HCFA, 1992). For each test system, the laboratory must follow the manufacturer's test system instructions, using calibration materials provided or specified, and with at least the frequency recommended by the manufacturer. If reference materials are not provided by the manufacturer (e.g., ASR or in-house-developed tests), the laboratory must use calibration materials appropriate for the test system (method- and matrix-appropriate target values) and, if possible, traceable to a reference method or reference material of known value

(HCFA, 1992; CAP, 2007). Calibration materials should include at least a minimal (or zero) value (lower limit of reportable range), a midpoint value, and a maximum value near the upper limit of the range to verify the laboratory's reportable range of test results. The laboratory protocol must include the required frequency of calibration, the number, type, and concentration of calibration materials, and the acceptable limits. The recalibration of non-FDA-cleared systems should be performed, at a minimum, (i) when recommended by the manufacturer; (ii) after major instrument service, maintenance, or replacement of critical parts; (ii) at least every 6 months; (iv) if system failure is detected by unacceptable QC performance and investigation and correction fails to correct the problem; and (v) at changes of reagent lots, unless the laboratory can demonstrate that the use of different lots does not affect the accuracy of patient or client test results and the range used to report patient or client test data (HCFA, 1992; CAP, 2007). Environmental conditions, including temperature, humidity, and/or carbon dioxide measurements, are recorded daily, with measurement verified periodically by external standards.

For reagents and kits, the frequency, number, and types of controls required are dependent on several parameters, including the designated complexity of the test (CLIA waived or low, moderate, or high complexity) and the presence of an internal performance control. CLIA requirements for the types and frequency of testing are listed in Table 7. At a minimum, QC must be performed with each new lot and new shipment (including the same lot). The laboratory is required to follow the most stringent requirement, whether it is from the manufacturer of the test, CLIA, or supplemental regulatory agencies, such as CAP, JCAHO, or local, state, or federal accrediting agencies. For each test system, the laboratory is responsible for having control procedures that monitor the accuracy and precision of the complete analytical process. The laboratory must establish the number, type, and frequency of testing control materials using, if applicable, the performance specifications verified or established by the laboratory. In addition, control material must be tested when new lots of reagents are used, a major preventive maintenance is performed, or any critical part of an instrument that may influence test performance is replaced. If unassayed control materials are used, then the laboratory must establish statistical parameters for the expected result and/or result ranges. Once this range has been established, concurrent testing of new control materials can be validated by comparison to the previously determined statistical parameters.

All reagents, media, solutions, stains, antisera, and kits must be labeled with the identity, concentration, reactivity, purity (or sterility), storage conditions, source, safety hazard information, and dates of preparation, receipt, use, and expiration (Warford, 2000). Reagents and kits must not be used when they have exceeded their expiration date, have deteriorated, or are of substandard quality. Components of reagent kits of different lot numbers must not be interchanged unless otherwise specified by the manufacturer (HCFA, 1992; CAP, 2007). Each new reagent, kit lot, or shipment must be tested in parallel with a prior product of satisfactory performance or appropriate external control material. If appropriate external control material is not available, then nonexpired kit controls from a previous lot can be used to validate the performance of the new lot or shipment.

For direct antigen tests (such as those for influenza virus and RSV) that do not contain an internal performance control, a positive control for each antigen detected and a

TABLE 7 CLIA requirements for QC: types and frequency[a]

For each test system, patient specimens are assayed or examined at least once each day

- For each quantitative procedure, include two control materials of different concentrations
- For each qualitative procedure, include negative and positive control material
- For test procedures producing graded or titered results, include a negative control material and a control material with graded or titered reactivity, respectively
- For each test system that has an extraction phase, include two control materials, one that is capable of detecting errors in the extraction process and one for the performance phase
- For each molecular amplification procedure, include two control materials and, if reaction inhibition is a significant source of false-negative results, a control material capable of detecting the inhibition

For each electrophoretic procedure, include concurrent with patient specimens, at least one control material containing the substances being identified or measured

For reagent, media, and supply checks, the laboratory must do the following

- Check each batch (prepared in-house), lot number (commercially prepared), and shipment of reagents, disks, stains, antisera, and identification systems (systems using two or more substrates or two or more reagents, or a combination) when prepared or opened for positive and negative reactivity as well as graded reactivity, if applicable
- Each day of use (unless otherwise specified in this subpart), test staining materials for intended reactivity to ensure predictable staining characteristics; control materials for both positive and negative reactivity must be included, as appropriate
- Check fluorescent and immunohistochemical stains for positive and negative reactivity each time of use
- Before, or concurrent with the initial use
  - Check each batch of media for sterility if sterility is required for testing
  - Check each batch of media for its ability to support growth and, as appropriate, select or inhibit specific organisms or produce a biochemical response
  - Document the physical characteristics of the media when compromised and report any deterioration in the media to the manufacturer
- Follow the manufacturer's specifications for using reagents, media, and supplies and be responsible for results

[a]Source: HCFA, 1992.

negative control must be done each day of testing (HCFA, 1992; CAP, 2007). If the assay requires an antigen extraction step, the positive control used must be appropriate for detection of problems that may occur in the extraction step. If the direct antigen test does contain an internal performance control (including flow or procedural controls), then an external positive control(s) (organism or antigen extract for each antigen tested) and negative control must only be run with each new kit lot or separate shipments of a given lot number (HCFA, 1992; CAP, 2007). For tests with an extraction step or those that are labeled by CLIA as high complexity, then the system must be checked each day with a positive organism for each antigen tested. For DFA screening directly on clinical specimens (e.g., respiratory cells or skin vesicles) or for virus identification from cell culture, testing must include a positive and a negative control for each virus tested. If pooled fluorescent antibodies are used for primary screening, the pools should be validated before use by testing against the individual viruses detected by the pool. Confirmation of reactivity with a pooled control DFA reagent is not sufficient, as individual virus reactivity has not been confirmed. After verification of acceptable performance, daily testing using one of the viruses detected by the pool is acceptable, and it is recommended that the viruses detected are rotated as controls on a regular basis (Warford, 2000).

Three control levels, reactive or high titer, weakly reactive or low titer, and negative, are required for quantitative assays, plus standards or calibrators, as specified by the verified method (HCFA, 1992; NCCLS, 2003; CAP, 2007). In addition to the controls that are part of the test kits, the use of external controls from a different source, such as CAP or commercial suppliers, has been reported to be valuable in detecting random and systematic errors in testing (Gray et al., 1995a, 1995b; Yen-Lieberman et al., 1996). External controls of a known value are essential for quantitative assays to monitor lot-to-lot variance of reagents that can affect quantitation and also the limit of detection (sensitivity of the assay). This is of particular importance for assays such as human immunodeficiency virus type 1 viral load, where patient monitoring over time is critical to assess therapeutic success or failure. New lots of reagents that cannot reproducibly give the expected quantitative result (within an acceptable variance) for external standards as with previous lots should not be accepted by the laboratory. Failure to document positive and negative control results and instrument function tests and preventive maintenance are among the top CMS and CAP inspection deficiencies cited (Table 2).

Red cell suspensions used for quantitative serologic procedures must be standardized (HCFA, 1992; CAP, 2007). Criteria for the degrees of agglutination and lysis for quantitative assays must be defined by the laboratory to ensure standard reporting of results. Worksheets or records must indicate, when known, the actual titers of reagents and control sera. Reactive and nonreactive controls must be included in the serologic detection of viral antibodies.

Cell Culture QC

The laboratory must have sufficient cell types available to allow for the recovery of the range of viruses, potentially present and detectable in cell culture, for specimen types processed by the laboratory (HCFA, 1992; Warford, 2000; CLSI, 2006e; CAP, 2007). Cultures must be incubated under the appropriate conditions (temperature and atmosphere) and for an optimal length of time to permit isolation or detection of all viruses for which services are offered. Some accrediting agencies, such as CAP, specify the types of cells and incubation periods for isolation of representative viruses (CAP, 2007). Cell culture systems can, however, be one of the most variable test methods used in the clinical laboratory and, therefore, can be susceptible to various conditions that can adversely affect results. For example, the ability of a virus to propagate in a cell line can be affected by the cell culture source and lineage, number of passages, age and condition of the monolayer, adverse conditions during shipping, and presence of contaminating agents (CLSI, 2006e). Therefore, very specific test guidelines and QC procedures must be followed to maintain a high standard of performance.

QC requirements specific for viral isolation are listed briefly in Table 8 (Miller and Wentworth, 1985; HCFA, 1992; Lennette, 1995; Clarke, 2004; CLSI, 2006e) and include maintaining cell culture records with receipt date, cell types, source, passage number, lot number, age of cells, container type (tube, shell vial, or cluster plate), and media used for their growth and maintenance. The condition of vessels upon receipt should be noted, and specific mention should be made of leaking or cracked vessels. The laboratory should verify that the manufacturers of commercially prepared cell lines perform studies to ensure the susceptibility of cell lines, provide for ongoing monitoring of cell lot performance, and make all QC data available upon request. Laboratories that prepare their own cell culture vessels must have well-documented policies, procedures, and verification data that the selected cell lines are suitable for the isolation of the intended virus(es), that there is ongoing QC for monitoring cell susceptibility, and that cell lines remain free of contamination.

For optimal performance, cells should be inoculated within 7 days of receipt (8 to 10 days of seeding), with monolayers at 75 to 90% confluence (CLSI, 2006e). Confluent or overgrown monolayers may obscure viral cytopathic effect (CPE)

TABLE 8 Cell culture-specific QC documentation[a]

Cell culture records with cell types, passage number, source, age (<10 days postseed) and receipt dates, and maintenance media (each lot and date)
Uninoculated cells of each lot maintained as negative controls for mycoplasma contamination, cytopathology (CPE), and nonreactivity in immunofluorescence, hemagglutination, or hemadsorption identification assays
Cell cultures are available and incubated appropriately for isolation or detection of entire range of viruses for which services are offered
Inoculated cell cultures checked at least every other day for initial 14 days of incubation
Culture media and additives checked for sterility, growth promotion, and absence of toxicity
Antisera and antigens checked for positive and negative reactivity each day of use
Red blood cell suspensions checked by spectrophotometer periodically
Buffers and diluents checked for pH, sterility, and absence of cytotoxicity
Water quality: pyrogen- and bacterium-free type I water or superior
Biological safety cabinet appropriate for classification of viruses propagated, certified at least annually
Daily decontamination of hoods and benches with high-level disinfectant

[a]Source: Warford, 2000.

or adversely affect the recovery of some viruses, such as RSV. Documentation by the laboratory of in-house-prepared cell culture vessels or for those supplied by the manufacturer with each shipment should be retained. Cell cultures should be observed microscopically to confirm that the cells are attached to the substratum, the confluence of the monolayer is appropriate, and the cell appearance is typical. Cell culture media should be near a neutral pH (salmon pink in color) and free of contamination (clear). Cell cultures not meeting the expected criteria should be observed or rejected, depending on the observation. Continuous cell lines must be checked for mycoplasma contamination by the laboratory, or the vendor must supply documentation that this has been performed prior to shipment.

Each day that patient specimens are inoculated, uninoculated lot-matched tubes, shell vials, or cluster plates should be incubated, maintained (i.e., medium changes), and observed in the same manner as inoculated tissue cells. This is recommended as a negative control for CPE, mycoplasma contamination, toxicity, and identification methods such as hemadsorption and/or hemagglutination, and immunofluorescence. Primary cell culture endogenous agents, such as simian viruses 5 and 40, foamy retrovirus, herpesvirus B, or contaminants from media or additives, such as serum, can produce CPE, which may mimic or interfere with viral CPE and may cause hemadsorption with red blood cells or nonspecific fluorescence (Warford, 2000). The CLSI guidelines for viral culture also note that use of negative culture controls by each technologist each day viral inoculation is performed can be an effective way to monitor technologist performance and to help identify technical problems such as sample cross-contamination or reading errors (CLSI, 2006e). In addition to the negative controls, one or more vessels from each lot number should be incubated unopened so that if a problem should occur with the cell culture medium, the laboratory will be able to determine if the problem occurred with the manufacturer or locally at the laboratory (CLSI, 2006e). Although the use of negative controls is beneficial, the laboratory must keep in mind that this type of screening can be limited and adverse effects may occur in other portions of the cell culture lot. Any suspected problems with cell culture systems should be promptly investigated, documented, and immediately reported to the vendor.

The use of daily positive controls to monitor traditional tube culture performance is not routinely performed. However, many laboratories select commonly isolated viruses to test each cell line upon receipt in the laboratory. Positive controls serve as a source of control material for detection and confirmation methods such as DFA and hemadsorption and are useful for training and competency assessment. Positive controls are also useful in comparing the susceptibility of cell lines from various vendors and for a continued assessment of the quality of the cell lines to support the growth of various viruses. Unfortunately, due to the nature of cell culture, most QC is performed simultaneously with use for patient testing, and problems are often noted in a somewhat retrospective manner. Despite this problem, cell culture performance and cell line sensitivity should be monitored regularly. This can be achieved in several ways, including the comparison of viral titrations or by comparison of culture detection rates to historical data. Although the overall rate of detection of specific viruses can vary significantly from season to season, comparison to other methods, such as DFA or the same enzyme immunoassay (EIA) method, should remain relatively comparable. For example, if historical data indicate that the sensitivity of viral culture for RSV is 75% compared to 85% for DFA, a loss of culture sensitivity would indicate a potential problem with either culture methods, cell lines, or poor technical performance by laboratory staff.

Daily target-specific positive controls are, however, required for test systems that do not use CPE but viral antigen detection using immunostaining or fluorescent antibodies to identify the infecting virus (HCFA, 1992; CAP, 2007). This would include blind staining of shell vial cultures for either single viruses or multiple viruses. When a single system (for example, R-Mix respiratory virus culture, Diagnostic Hybrids, Athens, OH) can detect more than one virus (influenza virus A, influenza virus B, adenovirus, parainfluenza types 1, 2, and 3, and RSV), daily positive virus isolate controls can be rotated so that, during the course of 1 week, the lot of R-Mix and the DFA pool have been tested against all viruses routinely isolated. However, the DFA pool reagent must be initially tested with all viruses individually to confirm reactivity prior to use in this test system.

The maintenance medium lot and date of use for each cell culture should also be recorded. Buffers, media, and additives should be checked for pH, sterility, growth promotion, and absence of toxicity in cell culture. Water that is used with cell culture should be pyrogen- and bacteria-free type I water or superior as defined by CLSI (CLSI, 2006d). To prevent cell culture contamination and to ensure the safety of staff, virology benches and safety cabinets should be disinfected at least daily with a high-level disinfectant, such as 10% sodium hypochlorite (bleach). A class II or higher biological safety cabinet with HEPA filters and, if possible, external venting, certified annually, at a minimum, plus facilities and procedures that are appropriate to the biohazard level of the viruses tested as defined by the CDC must be used for virus isolation (CDC, 2007).

Molecular Testing QC

QC procedures that are specific for molecular testing are listed in Table 9 (AMP, 1999; NCCLS, 2003, 2004b; CLSI, 2006a, 2006c, 2007b). The number and types of controls should be determined based upon the requirements or recommendations of the manufacturer (FDA-cleared assays, IUOs, and RUOs), CLIA, CAP, and regulatory agencies. Optimal QC is achieved with the use of the sample internal controls as well as the other external controls that are handled the same as patient samples, including the initial steps of specimen processing and continued through all subsequent steps, amplification, separation, hybridization, and detection. Internal controls that are added to each sample prior to nucleic acid extraction are an excellent way to monitor all components of the assay, including nucleic acid extraction, removal of inhibitory substances, reagent integrity, amplification and detection efficiency, and technical performance. Internal controls should be added at a relevant concentration so they are not detected when low levels of inhibitors are present in the sample that could lead to false-negative results, especially when targets may be present at low concentrations. For assays without an internal control, documentation can be provided by the manufacturer (FDA-cleared assays) or the laboratory must have sufficient validation data to demonstrate that the inhibition rate falls within an acceptable range. Inhibition controls are not required for assays that that use probe-based hybridization methods (CAP, 2007).

Minimally, positive and negative external controls, preferably prepared in the same matrix (or matrices) as the clinical samples tested, should be tested in parallel with patient samples each day of testing (CAP, 2007). However, external positive and negative controls are not required each day of

TABLE 9 Molecular testing: specific QC documentation[a]

Amplified DNA testing procedures must prevent nucleic acid cross-contamination by:
• Separate reagent preparation, specimen preparation, and amplification/detection areas
• Dedicated equipment for each area, including lab coats, pipettes, aerosol barrier pipette tips, and powder-free disposable gloves
• Unidirectional workflow from clean (DNA free) to dirty (amplified DNA) areas
• Incorporation of contamination prevention chemicals such as psoralens and/or uracil-*N*-glycosylase
• Parallel testing to evaluate DNA inactivation
Primers and probes: known sequence, concentration, purity, parallel testing of each lot
Positive and negative controls for all phases of testing: specimen processing, amplification, hybridization, and detection
• Additional recommended controls include reagent blank and internal control for inhibition
• Molecular weight standards for electrophoresis (each run)
Thermal cyclers, water baths, heat blocks, and incubators: follow manufacturer's instructions for use, preventive maintenance, and service; perform calibration at least yearly, or after major service; and monitor temperature and function (each day of use)
Spectrophotometers, luminometers, and pipettes: follow manufacturer's instructions for use, preventive maintenance, and service; perform calibration at least every 6 mo; and monitor function (each day of use)

[a]Source: Warford, 2000.

testing for single-test-unit enclosed systems that contain an internal monitor for the entire process, including extraction, amplification, and detection. For such test systems, the testing of external positive and negative controls must be performed for each new lot and/or shipment or more frequently if required by the manufacturer (CAP, 2007). Ideally, the external positive control should contain a low level of target and thus serve simultaneously as a monitor of assay sensitivity. For quantitative assays, CLSI and CAP recommend that a negative control and 2 levels (low and high) of positive controls are tested with each run. The low-level control should also serve as a sensitivity control that would detect fluctuations in assay performance that could adversely affect the detection of low-level virus concentrations in patient samples. This is especially critical for samples, such as cerebrospinal fluid, where viruses often are present at very low levels. In addition to external controls, the appropriate standards, required to generate a quantitation curve, must be run, unless a reference calibration has been performed and verified. Reverification of the calibration curve must be performed as described in the section on validation and verification.

Prevention of contamination is also critical to QA in molecular testing due to the sensitivity of molecular amplification assays. Laboratories must adhere to and monitor compliance to their policies to prevent cross-contamination of samples (CAP, 2007). Cross-contamination can occur during sample processing, nucleic acid extraction, the addition of sample to reagents, and postamplification. CLSI suggests the use of chemicals, such as uracil-*N*-glycosylase and isopsoralens for contamination control (NCCLS, 2003). Environmental controls such as plugged pipette tips, frequent glove changes, use of dead air boxes, and the decontamination of work surfaces with compounds that degrade potentially cross-contaminating nucleic acids are also suggested. Laboratories that use conventional amplification methods that require the opening of tubes containing amplified products should adhere strictly to practices that mandate separate equipment and rooms for pre- and postamplification steps. Wipe tests can be performed on a scheduled basis to monitor surface contamination or performed when possible cross-contamination is noted in test runs. Gross contamination can be monitored using the negative control. However, it is often difficult to detect rare sample-to-sample carryover that can lead to false-positive results. Indicators of potential contamination can include the observance of low-level positive samples adjacent to high-level positive samples and an increase in the standard frequency of positive results per number of samples tested.

Reagents, particularly the oligonucleotide primers and probes, must be of known sequence, concentration, and purity and prepared using good manufacturing practices. The QC requirements for instruments, such as thermal cyclers, spectrophotometers, and luminometers, include functional tests and temperature monitoring each day of use (HCFA, 1992; CAP, 2007). Preventive maintenance and calibrations must be in compliance with manufacturer's instructions. Pipette calibration and temperature verification are critical to accuracy and reliability in molecular testing; CLSI recommends thermal cycler monitoring of at least 12 wells monthly (all wells over the course of 1 year), with a thermocouple and pipette calibration at least twice per year. Laboratories with high-volume testing should consider pipette calibration on a more-frequent basis. If the laboratory cannot verify the performance of an enclosed real-time amplification and detection system, then the system must be certified at least yearly by the manufacturer or in accordance with the manufacturer's guidelines.

Postanalytical Phase

The final part of testing, and often that which is most prone to human error, is the reporting of patient results. Accurate entry of results is best facilitated by direct instrument-to-LIS interfaces. Results are electronically transferred to work lists that must, however, be checked by laboratory staff for accuracy prior to verification of results and release. However, for most manual virology procedures and low-volume molecular assays, results must be entered into the LIS manually. Transcription errors can be a major source of poor laboratory performance. A system must be in place to rapidly detect and correct clerical errors and ensure that all electronic data are accurate and delivered to the final destination in a timely manner (HCFA, 1992; CAP, 2007). Items that should be verified periodically include (i) results reported from calculated data, (ii) results and patient-specific data electronically reported to network or interfaced systems, and (iii) manually transcribed or electronically transmitted results and patient-specific information reported directly or upon receipt from outside referral laboratories, satellite, or point-of-care testing locations.

The format of the result reporting must be concise and clear and provide all relevant information, including positive patient identification, the name and address of the laboratory location where the test was performed, test report date, test performed, specimen source, test result, and if applicable, the units of measurement or interpretation, or both, and reference values (normal ranges). When indicated, the report

should also include methodology, reportable range, sensitivity and specificity of the assay, interpretive criteria, and limitations of the test. Laboratories must acknowledge the use of non-FDA-cleared assays and which entity was responsible for establishing the performance characteristics of the test. Other notes might include information regarding the condition and disposition of specimens that do not meet the laboratory's criteria for acceptability. The laboratory must have in place a policy for the notification of critical, panic, or alert values. The laboratory must immediately alert the individual or entity who requested the test and document using a "read back" system that the result was delivered in a timely fashion (JCAHO, 1998). If testing is delayed, errors were identified, or unacceptable results obtained, the laboratory must notify the ordering entity in a timely fashion and issue, if indicated, a corrected report. Records and reports must be maintained for sufficient time as designated by federal, state, and local regulatory agencies.

LABORATORY DESIGN, SPACE, AND SAFETY

The design and space requirements of clinical virology laboratories will vary greatly depending on the level and types of viral diagnostic services offered and the biohazard level of the viruses handled (Jamison et al., 1996; CDC, 2007). Architectural firms that specialize in laboratory design should be used for guidance. Requirements for biosafety in virology laboratories are detailed in a CDC publication (CDC, 2007). Aside from the use of routine MEIA, the majority of viral diagnostic testing requires a biosafety level 2 (BSL2) facility. Exceptions that the routine clinical laboratory may have to consider include the handling of specimens from patients with the potential to contain avian influenza or the SARS coronavirus. For samples possibly containing avian influenza virus, antigen testing (DFA and MEIA) requires a BSL2 facility using BSL3 practices. Culture should not be performed, and specimens should be referred to the local Public Health Laboratory. Specimens suspected of the SARS coronavirus must be cultured only in a BSL3 facility. Both for viral isolation and viral molecular testing, it is recommended to maintain separate areas for reagent preparation and specimen processing, which are not contaminated with infectious viral agents or nucleic acid. A separate biological safety cabinet is desirable for "clean work" with uninfected cell culture, reagent, and media preparation and/or sample processing prior to culture or nucleic acid amplification. The "dirty" areas of positive culture identification and amplified nucleic acid detection are ideally located in separate rooms and/or safety cabinets. Universal safety precautions are required in all clinical laboratories and are particularly important in virology when handling infectious pathogens in high concentrations. Disposable gloves and gowns and frequent disinfection of work surfaces and biological safety cabinets with 10% bleach followed by 70% alcohol are important for both safety and prevention of contamination of the work areas.

EVALUATION OF REFERENCE LABORATORIES

Referral of esoteric and low-volume tests to an offsite laboratory still requires the referring laboratory to monitor the quality of the testing (NCCLS, 1998b). The laboratory must select a reference laboratory that meets state and federal regulations and provides quality services. CLSI suggests the following criteria for reference laboratory evaluation: proof of licensure and accreditation, use of FDA or validated assays, copies of validation studies and written protocols, proficiency test results, frequent internal and external QC use, appropriate test turnaround time, qualified laboratory staff, high-quality methods, instrumentation and facilities, specimen stability during transport, good result reporting systems, policies for resolving questionable results, and cost (Warford, 2000). Parallel testing of new assays, critical to patient management, with split samples may be advisable to verify the performance of the selected reference laboratory (Warford, 2000).

COMPREHENSIVE QA AND IMPROVEMENT

CLSI considers continuous quality improvement to have five integrated quality system components, including quality planning, quality teamwork, quality monitoring, quality improvement, and quality review (NCCLS, 2004a). QA and improvement of the overall testing process are mandated by CLIA-88 and are critical to accurate and reliable laboratory services. Mandatory QA documentation is listed in Table 10. However, requirements for the preanalytical and postanalytical phases are limited to assessing patient management, comparing test results performed by different methods, and correlating test results with patient information (Warford, 2000). Additional interventions to improve the quality of specimens tested and the time to reporting test results increase patient and physician satisfaction and address the majority of the problems affecting patient care reported by physicians (Nutting et al., 1996). Comparing results of new and different methods for identifying viral infections can be stimulating and rewarding for the clinical virology staff, especially when the test data are obtained in conjunction with patient clinical information. CAP has a program, called Q-probes, for comparing pre- and postanalytical indicators of quality among laboratories. Past indicators have included measures of turnaround time, reference laboratory quality, test order accuracy, and other critical issues but only one survey specific to virology, hepatitis test utilization. QA has been challenged as too costly and of unproven benefit, but several studies have demonstrated that the costs of the QC and QA are low compared to the costs of quality failures—erroneous test results, repeated tests, recollected samples, and delays in test reporting (Westgard et al., 1984; Bartlett et al., 1994; Farrington et al., 1995; Warford, 2000). Based on a review and analysis of many published QC procedures and studies, Bartlett emphasizes the need for continuous quality improvement processes. Quality management guidelines, with a focus on continuous quality improvement, addresses the JCAHO requirement to demonstrate programs for monitoring, intervention and improvement of quality indicators affecting patient outcomes (JCAHO, 1998). Although the quality indicators clearly linked with improved patient outcomes are still to be established, such patient outcome studies will be the ideal performance measures of laboratory quality.

QA OVERSIGHT

A main component of monitoring laboratory QA has been the process of site inspections (surveys) that are conducted in accordance with CLIA survey requirements. In June 2006, the U.S. Government Accountability Office (GAO) provided testimony on the oversight of the quality of testing performed by the nation's clinical laboratories (Aronovitz, 2006). The limited data available suggested that state survey

TABLE 10 Mandated QA documentation[a]

PT performed for all assays in the same manner and by the same staff as patient sample testing in routine workflow with lab director's review, investigation, and corrective action for failures

- Alternate QA program for assays and analytes without formal, graded proficiency samples to verify test accuracy and reliability, such as use of samples split with reference lab, standard reference materials, and/or evaluation of patient clinical outcomes

QC: monitoring, weekly supervisory review, and corrective action

- Equipment and instrument preventive maintenance and service according to manufacturer's instructions, function tests, and calibrations
- Temperature-dependent equipment monitored each day of use and thermometers verified with standard; carbon dioxide levels in incubators monitored daily and internal monitor verified weekly with Fyrite analyzer
- Reagents, media, stains, solutions, and antisera labeled with identity, concentration, storage conditions, source, safety hazard information, and dates of preparation, receipt, use, and expiration
- Reagent and media reactivity, analysis and/or purity and sterility, parallel testing (each lot), positive and negative control results (each day of use)
- Antisera and antigen titers, parallel testing (each lot), positive and negative controls (each day of use)
- Quantitative test controls of three levels: negative, reactive, and weakly reactive
- Internal controls, when indicated
- External (non-kit) control limits established (if used) to detect random and systematic error(s)

Procedure manual written in CLSI format reviewed annually by director including:

- Preanalytical steps of patient identification, sample collection, transport, and storage and specimen rejection policy
- Analytical methods with verification of performance claims or validation
  - Verify assay performance for accuracy, precision, reportable range, and reference range
  - Validate in-house assay performance by establishing accuracy, precision, analytical sensitivity and specificity, reportable range, and reference range with known positive and negative samples
- Postanalytic processes, including prompt and accurate reporting with provider notification of critical values, interpretation and charting of test results, and amended reports
- QA program for total testing process with active evaluation and improvement to detect and correct significant errors

Specimen log and test requisitions with complete patient information including diagnosis, infection type, source, and collection date and time

Staff: laboratory director, technical supervisor, testing personnel

- Written duties, responsibilities, and qualifications (education, experience, and licensure/certification)
- Ongoing training verification, performance evaluations, continuing education
- Annual competency and proficiency testing, color discrimination ability, and consistent microscopy detection and interpretation

[a]Source: Warford, 2000.

agency inspections do not identify all serious deficiencies. A lack of standardization among the survey agencies does not allow for CMS to effectively use these data to monitor laboratory quality, particularly since there are different definitions of a serious quality problem. The GAO reported that, based upon interviews with CMS and survey organizations, potential and real laboratory quality problems can be masked by survey, complaint, and enforcement weaknesses (Aronovitz, 2006). Five main areas of weaknesses in oversight are listed in Table 11. As a result of this testimony, significant changes have been made in the way oversight surveys are now conducted. Some of the major changes include (i) required training for inspectors; (ii) unannounced inspections; (iii) redefinition of the actual survey process to include reading, asking, and observing as the three methods for eliciting information during the inspection process; (iv) compliance with appropriately citing deficiencies; (v) ensuring that past deficiencies have been corrected; and (vi) providing information for laboratory personnel by anonymously reporting quality issues. Laboratories with high quality standards will welcome these changes and find that compliance is an ongoing component of their daily operational procedures.

TABLE 11 GAO-identified weakness in oversight programs

Balance between CLIA program's educational and regulatory goals is skewed toward education, resulting in an understatement of survey findings
Structure of survey teams may not ensure appropriate levels of training and the appearance of conflict of interest, which could undermine the integrity of the survey process
Anonymity and the laboratory worker's lack of knowledge on how to file a complaint suggests that some quality problems are not reported
Large numbers of laboratories with proposed sanctions that were never imposed led to serious deficiencies on consecutive surveys
Lack of timely determination by CMS of the continued equivalency of accrediting organizations and state exempt programs and review of inspection requirements before implementation

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Specimen Requirements: Selection, Collection, Transport, and Processing

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2

Laboratory diagnosis of virus infections has become more efficient in the past 15 years because of the development and implementation of molecular technology for routine use. In the early 1990s after the initial discovery of molecular amplification of target nucleic acids (PCR) a few years earlier, the technique was applied for the detection of herpes simplex virus (HSV) specimens from patients with central nervous system disease characteristic of this virus infection (Aslanzadeh et al., 1992; Mullis and Faloona, 1987; Rowley et al., 1990). At the Mayo Clinic, this procedure was performed in a dedicated facility designed to process specimens for molecular testing. This core laboratory contained physically separate and air-flow-controlled rooms for specimen processing and amplicon detection by conventional (gel electrophoresis and Southern blotting) methods. HSV is rarely isolated in cell cultures from cerebrospinal fluid (CSF) specimens; therefore, molecular detection of this viral infection became the new gold standard for laboratory diagnosis of this infection (Mitchell et al., 1997; Polage and Petti, 2006; Rowley et al., 1990).

The next level of molecular diagnosis in virology was initiated in the late 1990s by the availability of instrumentation (real-time PCR) which provided automation of nucleic acid target amplification and amplicon detection steps in a "closed system" (i.e., reaction tubes never opened during these steps). Importantly, this innovative technology provided for the integration of real-time PCR instrumentation into the routine clinical laboratory. Automated PCR platforms have generally replaced conventional amplification and detection of products by gel electrophoresis and Southern blot methods that frequently caused contamination events and false-positive results due to the inadvertent transfer of high-copy nucleic acid products to other specimens (Espy et al., 2006).

Cell cultures are required for only 5.7% of the total pathogens identified in our virology laboratory (Table 1). Importantly, 74.2% of the molecular tests are FDA approved (*Neisseria gonorrhoeae*, *Chlamydia trachomatis*, human papillomavirus [HPV]) or are analyte-specific reagents (HSV, varicella-zoster virus [VZV], cytomegalovirus [CMV], and Epstein-Barr virus [EBV]) which are commercially available.

New technology has expanded the selection, collection, and interpretation of test results compared to past years. Importantly, as test complexity and the sensitivity of test results increase, so too does the need for frequent and effective communication between the clinical service and the microbiology laboratory. This type of teamwork is necessary to provide each patient with the optimal sensitive and effective viral testing with the greatest efficiency and lowest cost.

Basically, the specimen requirements for cell culture- and molecular-based diagnostic tests are similar. We realize that the changing trend from the use of cell culture to molecular technology will be gradual over time and customized for each laboratory depending on clinical service needs. Our goal in this communication is to define unique and optimal specimen processing needs for each technology.

SPECIMEN SELECTION AND COLLECTION

The selection of an appropriate specimen is vital to a correct test result; this includes not only the source of the specimen but also the timing and volume of collections. Information pertaining to all of these issues should be collated into comprehensive tables listing general disease categories and associated viruses, including optimal specimen types, methods of collection, methods of detection, volumes required, and containers for transport (Table 2). This information should be updated for institutional procedure guides at least on an annual basis. These guides are essential for clinicians so that they can submit optimal specimens and interpret the results appropriately. Ideally, electronic ordering of laboratory tests will require that a specimen can be submitted only according to the appropriate conditions and correctly labeled so that the specimen is processed for optimal testing.

Other resources that should be provided to physicians are the positive and negative predictive values (PPV and NPV, respectively) of tests, when known. The clinician should be able to compare the pretest probability with the PPV or NPV to fully interpret the test result and then make an informed decision regarding therapy or further testing. For example, a test with 96% sensitivity for a disease with an incidence of 5% has a PPV of about 50%, whereas the same test sensitivity for a disease with a 20% incidence in the population would have a PPV of about 85% (Banoo et al., 2006). Clearly, the significance of the same test result can

TABLE 1 Molecular and culture-based tests[a]

Diagnostic method	Test	No. of samples	% of total
Molecular	High-volume tests		
	GC/CT[b]	95,274	
	HPV[b]	82,479	
	HSV/VZV[c]	57,238	
	CMV/EBV[c]	28,648	
	Subtotal	263,639	74.2
	Other molecular tests	71,463	20.1
	Total	335,102	94.3
Culture	All culture tests	20,377	5.7
Total		355,479	100

[a]Tests used in the Virology Laboratory, Mayo Clinic, 2006.
[b]FDA approved.
[c]Analyte-specific reagents.

vary in different populations. This type of analysis can be helpful in deciding the number or frequency of sampling for a particular test.

Similar to viral culture, the volume of sample necessary for molecular testing is usually 1 to 2 ml, but as little as 0.3 ml can be sufficient, depending on the techniques used in the laboratory. An important benefit is that once the nucleic acids are extracted from a sample, usually only a small portion (5 to 10 μl) is needed for a particular test. Thus, the same sample extract can be retested on the same or different assays should a physician have additional test requests.

Specimens should be collected early in the acute phase of infection. However, the duration of viral shedding depends on the type of virus and systemic involvement, as well as other factors, such as the age or immune status of the patient. For example, the median duration of viral shedding in nasal secretions detected by PCR was 11.5 days for respiratory syncytial virus (RSV) (37 patients) and 5 days for human metapneumovirus (MPV) (6 patients) in a pediatric population (von Linstow et al., 2006). Another study showed that experimental inoculation of RSV in healthy adults resulted in a mean shedding duration of only 4 days (Lee et al., 2004). Some viruses, such as West Nile virus (WNV), cause a short, low-level viremia and may be undetectable by the time of symptom onset. As a result, there may be several days between RNA detection and the first positive serology results. Thus, if WNV is in the differential diagnosis for a patient, it is important to submit specimens for both serology and molecular testing several days later if initial results for the specimens are negative (Ratterree et al., 2004; Tilley et al., 2006).

The significance of detection of a virus may have a different level of importance depending on the time of collection. For example, a CMV-positive urine specimen collected during the first 2 weeks of life from an infant with congenital disease is strong evidence supporting the viral etiology of the congenital anomalies, presumably since the agent was acquired in utero and active viral infection and replication of the virus are present. Conversely, isolation of CMV from the same source after 2 weeks would not discriminate between congenitally or postnatally acquired CMV infection.

Detection of a virus may occur in the absence of clinical symptoms, or during the prodromal phase of infection. For instance, HSV can be shed asymptomatically from multiple anatomic sites. In fact, using PCR as the detection method, some studies have shown carriers of HSV type 2 to shed 20 to 25% of all days (Sacks et al., 2004). Similarly, several days before a recurrent outbreak of HSV lesions, the site may itch or burn, and the sensitivity of PCR testing allows for detection of HSV shedding during this time. Prompt administration of an antiviral can shorten or prevent the formation of new lesions (Anderson, 2005).

The immunocompetence of a patient with a viral illness has a significant effect on the time and duration of virus excretion. For example, HSV is detected in specimens from lesions of immunocompromised patients for more than 21 days, while the mean duration of HSV shedding from immunocompetent men and women with genital infection is 11.4 days (Corey et al., 1983). New lesions due to VZV infection in immunologically healthy children develop over a 4-day period while new lesions form in most immunocompromised children for more than 5 days. Immunocompromised adults with zoster infection shed virus for a longer period (7.0 days) than otherwise healthy adults (5.3 days). In addition, zoster is much more likely to disseminate cutaneously in immunocompromised hosts than in immunocompetent hosts (Balfour, 1988). The presence of a virus in a specimen may depend on the source of the sample. Generally, VZV can be cultured from lesions for up to 7 days after the initial vesicles appear but from blood by culture or nucleic acid amplification only during the late incubation period or days 1 to 4 of the acute illness (Mainka et al., 1998).

Indeed, the type and number of positive specimens may be important to the interpretation of the results. For example, in a study of adenovirus in bone marrow transplant recipients, the number of sites was correlated with outcome. Adenovirus cultured from a single site indicated a 10% risk of clinical disease, virus detection from two sites indicated a 40% chance of clinical disease, and detection from three or more sites suggested disseminated infection and a low chance of survival (Carrigan, 1997). Interestingly, the same study also found that pediatric patients were three times more likely to be infected than adults, and the onset time was within 30 days posttransplant, whereas adults were typically infected after 90 days.

SOURCES

Respiratory Specimens

Specimens from the respiratory tract can represent almost one-half of the source material and one-third of the total

TABLE 2 Specimen information for diagnostic virology services[a]

General disease category	Virus	Specimen(s) used for:				Other comments
		Nucleic acid testing		Serology	Culture	
		Specimen(s)	Reference(s)			
Respiratory (pharyngitis, croup, bronchitis, pneumonia)	Influenza virus	Nasal or NP swab, NP washings or aspirate, throat swab, BAL fluid, sputum	van Elden et al., 2001; Boivin et al., 2003; Smith et al., 2003; Frisbie et al., 2004; Boivin et al., 2004; Espy et al., 2006	Serum	Nasal or NP swab, NP washings or aspirate, throat swab, BAL fluid, sputum	
	PIV	Nasal or NP swab, NP washings or aspirate, throat swab, BAL fluid, sputum	Templeton et al., 2004		NP aspirate, throat wash, throat swab, BAL fluid, sputum	
	RSV	NP washings or aspirate, NP or throat swab, induced sputum	Borg et al., 2003; Falsey et al., 2003; Gueudin et al., 2003; Hu et al., 2003; van Elden et al., 2003; Boivin et al., 2004		NP washings or aspirate; NP swab (Calgiswab)	Washings or aspirate preferred
	MPV	NP aspirate	Cote et al., 2003; Mackay et al., 2003; van den Hoogen et al., 2004			This virus is not routinely cultured
	Enterovirus	Throat swab, BAL fluid	Corless et al., 2002; Watkins-Riedel et al., 2002; Cote et al., 2003		Throat swab, BAL fluid	
	SARS-CoV	Tracheal aspirate, NP aspirate, throat or nasal swab, throat washings, stool	Ng et al., 2003; Chan et al., 2004; Drosten et al., 2004; Poon et al., 2004			Culture not recommended; tracheal aspirate or stool preferred
	HSV	Respiratory washings, BAL fluid, throat swab, tissue	Espy et al., 2006, and references therein		Respiratory washings, BAL fluid, throat swab, tissue	
	Adenovirus	Throat swab	Houng et al., 2002; Gu et al., 2003; Heim et al., 2003; Lion et al., 2003; Faix et al., 2004; Lankester et al., 2004; Leruez-Ville et al., 2004	Serum	Throat swab	
	CMV	Throat washings, blood, plasma, urine, ocular swab, PBLs, amniotic fluid	Espy et al., 2006, and references therein (for both qualitative and quantitative tests)		Respiratory washings, throat swab, BAL fluid	

Exanthem (maculopapular)	Adenovirus	Throat swab	Houng et al., 2002; Gu et al., 2003; Heim et al., 2003; Lion et al., 2003; Faix et al., 2004; Lankester et al., 2004; Leruez-Ville et al., 2004	Serum	Throat swab	
	Enterovirus	Throat swab, rectal swab	Corless et al., 2002; Nijhuis et al., 2002; Watkins-Riedel et al., 2002		Throat swab, rectal swab, stool	
	Rubella virus			Serum, CSF		Only one serum specimen is required for determination of immune status to rubella virus; for evidence of acute infection (rubella or measles), 2 or more serum specimens are needed, taken 2–3 wk apart, unless IgM antibody result is positive in first specimen
	Measles virus (rubeola)			Serum		
	Less frequently found viruses					
	PIV	NP aspirate or washings, throat swab, throat washings, BAL fluid	Borg et al., 2003; Falsey et al., 2003; Gueudin et al., 2003; van Elden et al., 2003; Templeton et al., 2004; Boivin et al., 2004	No serology for PIV	NP aspirate or wash, throat swab, throat wash	
	RSV	NP washings or aspirate, NP or throat swab, induced sputum		Serum	NP swab (Calgiswab), BAL fluid	
Exanthem (vesicular)	HSV	Dermal swab, genital swab, throat swab	Espy et al., 2006, and references therein	Serum	Dermal swab, genital swab, throat swab, vesicle scrapings for direct FA test	Vesicle scrapings for direct FA test: place a drop of saline in each of two separate areas of a glass slide 5–10 mm apart; transfer skin scrapings from a scalpel blade to the saline and spread the cells over a small circular area (5–10 mm in diameter)
	VZV	Dermal swab	O'Neill et al., 2003; van Doornum et al., 2003; Schmutzhard et al., 2004	Serum	Dermal swab, vesicle scrapings for direct FA test	
CNS (aseptic meningitis and encephalitis)	HSV	CSF, throat swab, tissue, dermal swab	Espy et al., 2006, and references therein	CSF, serum	Brain biopsy specimen, dermal swab	Culture of CSF has low sensitivity

(Continued on next page)

TABLE 2 Specimen information for diagnostic virology services[a] *(Continued)*

General disease category	Virus	Specimen(s) used for:				Other comments
		Nucleic acid testing		Serology	Culture	
		Specimen(s)	Reference(s)			
CNS (aseptic meningitis and encephalitis)	Enterovirus	CSF, throat swab, serum (infants, 1–2 ml), stool	Verstrepen et al., 2001; Aberle and Puchhammer-Stockl, 2002; Corless et al., 2002; Kares et al., 2004; Monpoeho et al., 2002; Nijhuis et al., 2002; Watkins-Riedel et al., 2002; Verboon-Maciolek et al., 2003		CSF (1–2 ml), throat swab, rectal swab, serum (infants, 1–2 ml), brain biopsy specimen (if available)	
	Arboviruses: California (LaCrosse), St. Louis encephalitis, Western equine encephalitis, Eastern equine encephalitis (see below for WNV)			Serum, CSF		These viruses are not routinely cultured; PCR assays are not widely available
	WNV	Blood, plasma, serum, CSF, body fluid	Briese et al., 2000; Lanciotti et al., 2000; Shi et al., 2001; Huang et al., 2002; Harrington et al., 2003; Hiatt et al., 2003; Sampathkumar, 2003; Cockerill and Smith, 2004; Namba et al., 2005	Serum, CSF		This virus is not routinely cultured; viremia may be brief; therefore, a negative molecular test should be followed by serology
	Dengue virus	Serum	Laue et al., 1999; Callahan et al., 2001; Houng et al., 2001; Shu et al., 2003	Serum, CSF		This virus is not routinely cultured; viremia may be brief; therefore, a negative molecular test should be followed by serology
	EBV	Plasma, blood, serum, PBMC	Espy et al., 2006, and references therein (for both qualitative and quantitative tests)	Serum		This virus is not routinely cultured

	VZV	CSF, dermal swab	Aberle and Puchhammer-Stockl, 2002; O'Neill et al., 2003; Stocher et al., 2003; van Doornum et al., 2003; Weidmann et al., 2003; Schmutzhard et al., 2004; Espy et al., 2006		CSF (1–2 ml)	
	CMV	CSF, amniotic fluid	Espy et al., 2006, and references therein (for both qualitative and quantitative tests)		CSF (1–2 ml)	
	Measles virus (rubeola)			Serum, CSF		Viral culture generally successful
	Rabies			Serum		Contact the CDC for further testing information
	Mumps	CSF, throat swab, urine	Uchida et al., 2005; Krause et al., 2006	Serum, CSF	CSF, throat swab (Stensen's duct), urine	
Infectious mononucleosis	EBV	Blood, PBLs, plasma	Espy et al., 2006, and references therein (for both qualitative and quantitative tests)	Serum		This virus is not routinely cultured; immunofluorescence test for antibodies to EBV indicated in those patients with heterophile-negative (rapid test method) determinations
	CMV	Plasma, blood, respiratory washings	Espy et al., 2006, and references therein (for both qualitative and quantitative tests)	Serum	Urine (5–10 ml), throat swab	
Hepatitis viruses	HAV	Serum	Costa-Mattioli et al., 2003; Abd El Galil et al., 2004; Brooks et al., 2005; Jothikumar et al., 2005; Costafreda et al., 2006; Sanchez et al., 2006	Serum		EBV and CMV occasionally cause hepatitis, especially in immunocompromised patients
	HBV	Serum	Qualitative and quantitative assays commercially available	Serum (for antigen or antibody tests)		
	HCV	Serum	Qualitative and quantitative assays, and sequence analysis, all commercially available	Serum		
	HDV	Serum		Serum (for antigen or antibody tests)		This virus is not routinely cultured
	HEV	Stool, serum	Mansuy et al., 2004; Orru et al., 2004	Serum		This virus is not routinely cultured

(Continued on next page)

TABLE 2 Specimen information for diagnostic virology services[a] *(Continued)*

General disease category	Virus	Specimen(s) used for:				Other comments
		Nucleic acid testing		Serology	Culture	
		Specimen(s)	Reference(s)			
Immunodeficiency virus	HIV-1, HIV-2	Plasma	Qualitative, quantitative, and genotypic analysis, all commercially available	Blood (various antigen and antibody assays)		
Gastroenteritis	Rotavirus			Stool (EIA)		Rotavirus in stool specimens detected by EIA
	Norwalk virus and noroviruses			Stool (EIA)		Assays for antibodies to Norwalk-like agents are performed in only a few research laboratories
	Astrovirus			Stool (EIA)		Astrovirus in stool specimens detected by EIA
	Adenovirus	Rectal swab, stool	Houng et al., 2002; Gu et al., 2003; Heim et al., 2003; Lion et al., 2003; Faix et al., 2004; Lankester et al., 2004; Leruez-Ville et al., 2004	Stool (EIA)	Stool (5 g) or rectal swab	Few "high numbered" serotypes of adenoviruses have been associated with gastroenteritis
Genital infections	HSV	Genital swab, dermal swab	Espy et al., 2006, and references therein	See comment	Vesicle swab, vesicle scrapings (see note for HSV in exanthem section)	Serology is rarely informative, unless used to detect primary genital infection
	HPV	Cervical brushes, cervical swabs, cervical biopsy specimens	Commercially available methods		Endocervical swab, biopsy tissue	An FDA-approved molecular test is available (Murray et al., 2007); HPV can be detected in Papanicolaou-stained cells; some biotypes of low- and high-risk viruses can be detected by commercial nucleic acid detection kits
Congenital infections	CMV	Plasma, blood, urine, ocular swab, amniotic fluid, respiratory washings	Espy et al., 2006, and references therein (for both qualitative and quantitative tests)	Serum	Urine (5–10 ml), throat swab	
	HSV	CSF, dermal swab, respiratory washings	Espy et al., 2006, and references therein	Serum	Vesicle swab, throat swab, amniotic fluid	Presence of IgM to CMV or HSV in cord or neonatal blood indicates congenital infection

	Rubella virus			Serum, CSF		This virus is not routinely cultured; IgM antibody to rubella should be assayed using serum from babies up to 6 mo of age; IgG antibody should not be determined, since its presence only reflects passive transfer from mother
	Enterovirus	CSF, serum, throat swab	Verstrepen et al., 2001; Corless et al., 2002; Monpoeho et al., 2002; Nijhuis et al., 2002; Watkins-Riedel et al., 2002; Verboon-Maciolek et al., 2003; Kares et al., 2004		Throat swab, serum (see comment)	Cord blood is a useful and productive specimen for recovering enteroviruses in cell culture in congenital infections
	Parvovirus B19	Synovial fluid, amniotic fluid, plasma	Aberham et al., 2001; Harder et al., 2001; Schmidt et al., 2001; Weimer et al., 2001; Hokynar et al., 2002; Knoll et al., 2002; Hokynar et al., 2004; Koppelman et al., 2004; Schorling et al., 2004	Serum		
Ocular infections	HSV	Corneal scraping, dermal swab, throat swab	Espy et al., 2006, and references therein	Serum	Swab (eye), vesicle swab, vesicle scrapings (see comment on HSV in exanthem section)	Serology may be useful only for the primary ocular infections due to HSV; specimens for PCR or culture are recommended
	Adenovirus	Throat swab	Houng et al., 2002; Gu et al., 2003; Heim et al., 2003; Lion et al., 2003; Faix et al., 2004; Lankester et al., 2004; Leruez-Ville et al., 2004	Serum	Swab (eye)	

[a]Abbreviations: NP, nasopharyngeal; PBLs, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cells; FA, fluorescent antibody; HAV, hepatitis A virus; HDV, hepatitis D virus; HEV, hepatitis E virus.

viruses diagnosed in the clinical laboratory. Although less than 6% of viral specimens are cultured at our institution, respiratory sources constitute the majority of those that are processed for culture (Table 1). Thus, to examine the question of the utility of culture in the future, the respiratory specimens provide the best paradigm. The two main arguments for maintaining culture include the isolation of unsuspected viruses and the ability to further characterize isolates. The latter is primarily in the domain of reference and academic laboratories and, in fact, can many times be done by molecular methods. The former will erode with the coming of multiplex molecular testing, where all common culturable (as well as some unculturable) respiratory viruses can be detected simultaneously in one specimen. Published studies over the past 10 to 20 years have clearly shown superior sensitivity of molecular methods above any other approach. Real-time PCR provides a dramatic increase in sensitivity for influenza A virus testing (45.7 to 121% increase) (Zitterkopf et al., 2006). Multiplex real-time PCR for influenza viruses A and B, RSV, and four serotypes of parainfluenza viruses (PIVs) had 30% more positive specimens than culture (Templeton et al., 2004). Our experience with molecular methods has shown that the recovery of respiratory viruses in general was increased to 16.5% from 9.2% of 557 specimens (Espy et al., 2006).

Additionally, rapid testing allows significant findings to be relayed to the physician and patient in a timely manner, and appropriate action can be taken to best improve the patient's outcome. For instance, one study showed that the results of a rapid-antigen influenza test had a significant impact on physician decision-making. Inappropriate antibiotic usage was decreased, while the correct medication (antivirals) increased (Falsey et al., 2007). Lastly, as more viral therapies become available, the utility of a viral identification is increasingly important, and faster is better.

The question of what specimen to use is an ongoing balance of sensitivity, patient comfort, and available equipment. Throat or nasal washings may be more productive for viral isolation than throat or nasal swabs, but few comparisons have been reported. One study found equivalent culture sensitivity for nasal swabs and nasal aspirates, except for RSV, where nasal aspirates were more sensitive (97% versus 76%) (Heikkinen et al., 2002). Aspirates and washings, however, require suction equipment, which may have limited availability in some settings. Another study found similar results using an immunofluorescence assay. In children less than 2 years old, nasopharyngeal aspirates were 30% more sensitive (97% versus 67%) than nasopharyngeal swabs for the diagnosis of RSV (Macfarlane et al., 2005). The study was based on 88 paired samples, with the paired specimens taken one from each nostril of the same child. Interestingly, the study also measured discomfort of the child, finding that the aspirate scored a 6 on an 8-point pain scale, and the swab scored a 5. On the basis of the children's distress, the parents preferred the swab (70%) to the aspirate (15%). Importantly, flocked rayon swabs exhibit superior performance characteristics for the collection and yield of viruses from respiratory tract specimens (Dunn et al., 2003). Regardless of the specimen collection method, various studies in which real-time PCR was used as the detection method have shown PCR to be 23.6 to 225% more sensitive than cell culture (Falsey et al., 2003; Gueudin et al., 2003; van Elden et al., 2003).

Rapid-antigen tests for influenza viruses A and B and RSV are made by several companies. Although their specificity can approach 80 or 90%, their sensitivity in published studies is generally only in the 80 to 90% range compared to culture (Leland and Ginocchio, 2007). This demonstrates that rapid-antigen assays have some utility for screening purposes, but the sensitivity can vary widely. Since the rapid-antigen tests are less sensitive than culture and culture is inferior to molecular testing, it stands to reason that the actual sensitivity of rapid-antigen tests is somewhat lower. Although at our institution, the PCR test costs twice as much as the rapid-antigen test, the cost correlates with approximately double the sensitivity.

Other respiratory specimens, such as sputum and bronchoalveolar lavage (BAL) fluid may also be productive specimens for viral testing. A recent study used induced sputum to test for influenza and RSV in asthmatic patients (Simpson et al., 2003). The results showed that PCR was superior to immunofluorescence antigen assays for both influenza (24% versus 1% positive, respectively) and RSV (37% to 20% positive). BAL fluid has largely replaced open lung biopsy for viral infections and can be used for the detection of CMV, PIVs, influenza viruses, and enteroviruses.

The respiratory tract has recently been a source for newly identified viruses, including human MPV, the severe acute respiratory syndrome (SARS) coronavirus (CoV), and the human bocavirus (HBoV). Although some can be cultured (taking several weeks), all are readily detectable by PCR. The SARS CoV is highly contagious and can produce severe symptoms in people of all ages. The other two viruses are being identified far more commonly and primarily in children. Newly described in 2001, MPV may account for 5 to 70% of hospitalizations of children (Kahn, 2003; van den Hoogen et al., 2004). The virus is in the family *Paramyxoviridae*, subfamily *Pneumovirus*, is related to RSV, and in fact, can produce similar symptoms. Another potential pathogen, HBoV, was identified in 2005 in Sweden using random primers and pooled respiratory samples (Allander et al., 2005). HBoV is a newly identified member of the *Parvoviridae* family. A study in the United Kingdom found that HBoV was found in 8.2% of 574 study subjects, the third most frequent finding behind RSV and adenovirus (Manning et al., 2006). In a population of children less than 2 years old, Kesebir et al. found that 0% of 96 asymptomatic children had detectable HBoV compared to 5% of those with respiratory symptoms (Kesebir et al., 2006).

As new viruses are identified and increasingly associated with disease, the number of respiratory specimens negative for a pathogen should decrease. For example, the Brisbane Respiratory Virus Research Study used 17 new PCR assays for viral pathogens to rescreen a collection of 315 respiratory specimens and found an increase in detection from 48 (15.2%) to 206 (67%) of the samples (Arden et al., 2006). The initial detection had employed shell vial assays, reverse transcription (RT)-PCR, and bacterial culture to detect RSV, PIV (types 1 to 3), influenza virus types A and B, adenoviruses, and bacterial pathogens. The newly detected viruses (numbers isolated are indicated in parentheses) included human herpesvirus 6 (11), rhinovirus (140), enterovirus (8), parechovirus (2), HBoV (15), and non-SARS CoV (11).

Dermal Lesions

HSV (70%), VZV (29%), coxsackievirus type A (1%), and perhaps, some echoviruses are the principal agents that can be cultured on a routine basis from dermal lesions. HSV replicates rapidly in cell cultures; nevertheless, maximum sensitivity for the detection of this virus generally requires 5 days postinoculation to detect all positive and negative

results. The shell vial cell culture assay, using virus-specific monoclonal antibodies in a fluorescence assay, reduces the time for HSV detection to 1 to 2 days postinoculation (Gleaves et al., 1985; Smith, 1983). Thus, all results (both positive and negative) can be reported the day following receipt of the specimen.

The ability to detect or isolate HSV in cell culture varies with the stage of the lesion. For example, HSV was recovered from 94% of vesicular lesions, 87% of pustular lesions, 70% of ulcers, and 27% of crusted lesions (Moseby et al., 1981). Similarly, smears prepared with cells obtained from vesicles for Papanicolaou, crystal violet, or immunofluorescence staining were superior to cells obtained from ulcers for the diagnosis of HSV infections. Skin biopsy of cutaneous lesions may be important in the diagnosis of systemic CMV and human herpesvirus 8, infections of immunocompromised patients (PCR detection) (Mendez et al., 1998; Swanson and Feldman, 1987). Importantly, the laboratory has an essential role in distinguishing HSV from VZV because the clinical presentation of the patient may not be typical for these specific viral infections.

Nucleic acid amplification has given a new dimension to the detection and management of central nervous system (CNS) diseases caused by HSV and, to a lesser extent, by VZV (Koskiniemi et al., 2002; Li et al., 2003; Mitchell et al., 1997). Automated instruments for performing real-time PCR in a closed system (amplification and detection of nucleic acid products performed without an opening reaction vessel) has allowed a rapid same-day turnaround time for reporting results (Espy et al., 2006; Schmutzhard et al., 2004; Stranska et al., 2004). The detection of HSV and VZV from dermal and genital specimens was highly significant in demonstrating the increased sensitivity and specificity of real-time PCR compared to shell vial cell culture assay (Espy et al., 2000a; Espy et al., 2000b; Fang et al., 1999). Similarly, VZV replicates rather poorly in cell cultures, but real-time PCR was 91% more sensitive than detection of the virus in shell vials and other diagnostic methods (Johnson et al., 2000; Lilie et al., 2002). Therefore, real-time PCR permits highly sensitive same-day detection and differentiation of both HSV and VZV from clinical specimens (Druce et al., 2002; Rubben et al., 1997; Safrin et al., 1997; Stranska et al., 2004). In our laboratory, this test has been performed up to six times each day since May 2000 and has replaced cell culture for the routine diagnosis of HSV and VZV (dermal) and HSV (genital) infections.

Blood

Detection of viruses from blood specimens provides evidence of disseminated, invasive infections which may lead to systemic disease. Early detection of CMV in blood specimens may trigger the institution of inductive antiviral therapy to prevent or limit the spread of the virus to target organs. In addition, monitoring of blood specimens for the presence of the virus provides laboratory evidence of antiviral efficacy (Kalpoe et al., 2005; Pillet et al., 2006; Sagedal et al., 2005; Watzinger et al., 2004).

CMV is essentially the exclusive agent recovered from blood specimens in cell culture assays. HSV is a rare cause of viremia; however, enteroviruses can be detected from serum from infants in the early neonatal period associated with septic infections (Dagan et al., 1985). CMV viremia has been presumed to be associated with peripheral blood leukocytes, especially in the early posttransplantation period of solid organ and bone marrow patients; however, anticoagulated whole blood could provide a blend of plasma or blood cells tested alone. Alternatively, whole-blood (anticoagulated) specimens are predictably toxic to cell cultures.

The diagnostic time for detection of CMV in conventional tube cell cultures (~8 days postinoculation) has been substantially reduced to 16 to 24 h postinoculation using the rapid shell vial assay, but overall, the sensitivity of cell culture methods has been poor compared to molecular diagnostic methods (Mengoli et al., 2004; Mori et al., 2000; Nitsche et al., 2003; Onishi et al., 2006). In addition, the shell vial assay is impractical for adaptation for use as a quantitative assay.

The antigenemia test, in which the 65-kDa lower matrix protein of CMV could be detected in polymorphonuclear cells in blood specimens, provided the first diagnostic laboratory method for quantitation of viremia due to this virus infection. Nevertheless, widespread acceptance and implementation of the antigenemia test in clinical laboratories has been restricted by the nonstandardized technical aspects of sample processing, demanding manual technical performance of the test methods, and subjective interpretation of results.

Both shell vial cell cultures (qualitative) and the antigenemia test (quantitative) have been shown to be less sensitive in comparative studies than molecular amplification by PCR (Smith, 2006).

Real-time PCR has been demonstrated to provide rapid, sensitive, and quantitative results for the detection of CMV from blood specimens. Molecular amplification of CMV DNA has technical characteristics superior to those of shell vial cell cultures and pp65 antigenemia for the diagnosis, prognosis, and assessment of antiviral therapy for viremia due to this virus (Espy et al., 2006; Smith, 2006).

PCR technology has also provided laboratory evidence of viremia for those viruses which are not recovered by cell culture methods. Typical examples include human immunodeficiency virus (HIV), hepatitis B and C viruses (HBV, HCV), polyomaviruses (BK virus [BKV]), parvovirus B19, and adenoviruses (Allain, 2000; Boeckh et al., 2005; Caruntu and Benea, 2006; Cavallo et al., 2003; Gallian et al., 2005; Santana et al., 2004; Schupbach, 2003).

CSF

Optimal recovery of enteroviruses by culture requires the use of Buffalo green monkey kidney and human rhabdosarcoma cell lines rather than the more traditional combination of human embryonic lung and primary cynomolgus monkey kidney cell lines (Dagan and Menegus, 1986; She et al., 2006). Of cultivatable viruses, nonpolio enteroviruses are frequently the only isolates from CSF. Although enteroviruses are common causes of CNS disease, laboratory diagnosis is frequently based on serologic findings or association with the recovery of a virus from this group at another anatomical site (Kupila et al., 2006). HSV is rarely cultured from CSF specimens and is generally associated with children with meningitis or adults with Mollaret's syndrome (Mitchell et al., 1997; Read, 2001).

The diagnosis of HSV CNS disease by molecular amplification became the new gold standard and prompted the revolution of molecular diagnostics (Rowley et al., 1990). This seminal finding was subsequently confirmed, and molecular amplification was used for the detection of other virus nucleic acids from CSF specimens, such as the other herpesviruses (CMV, VZV, EBV), HIV, and BKV (Aslanzadeh et al., 1992; Dong et al., 2005; Dugan et al., 2005; Stjernquist-Desatnik et al., 2006; Yamamoto and Nakamura, 2000). Importantly, this breakthrough technology

provided rapid diagnostic results early in the clinical course of disease so that appropriate antiviral therapy could be implemented (Debiasi and Tyler, 2004; Kennedy, 2005).

PCR amplification of enterovirus target nucleic acid has been demonstrated to be more productive than culture for the laboratory diagnosis of CNS infections due to these viruses (Hosoya et al., 2001; Verstrepen et al., 2001). Thus, for the two most common recognized causes of CNS (herpesviruses, enteroviruses), molecular amplification is considered the diagnostic test of choice (Kares et al., 2004; Kupila et al., 2006; Polage and Petti, 2006).

Other Sources

The use of feces as a specimen for virus culture has been reduced for cases of gastroenteritis with the realization that viruses that are noncultivatable in cell cultures (rotavirus, norovirus [caliciviruses], astroviruses, and perhaps some adenoviruses) are responsible for most cases of viral gastroenteritis (Christensen, 1989; Mautner et al., 1995; Mitchell et al., 1995). These viruses can, however, be detected using enzyme immunoassay (EIA), and some laboratory services are also developing molecular tests. It is estimated that rotaviruses are the most prevalent cause of gastroenteritis worldwide, and they account for 25 to 65% of severe gastroenteritis in infants (Goodgame, 2001). Approximately 3.5 million cases of symptomatic rotavirus infection occur in the United States each year. Fortunately, rotavirus vaccines should reduce the number and severity of illnesses, resulting in fewer office visits and hospitalizations. Enteroviruses can be isolated as commonly from the feces of individuals without disease as from patients with gastroenteritis. Therefore, in the case of CNS disease with a suspected enterovirus etiology, a sample of CSF should be submitted for analysis by PCR. In the event that a PCR test for enteroviruses is unavailable, a stool sample or rectal swab may be submitted for culture, but the significance of an enterovirus in this sample must be carefully interpreted. An emerging pathogen for which stool is an excellent specimen for RT-PCR detection is the SARS CoV (Lau et al., 2005; Louie et al., 2006; Tang et al., 2004).

Mumps, adenovirus, and CMV are commonly cultured from urine after symptoms develop, although urine is a better specimen for detection of CMV than for mumps. Urine specimens submitted for the laboratory diagnosis of CMV infection can be inoculated directly into cell cultures because the virus is not concentrated in urine sediment after low-speed centrifugation. Urine is the best single specimen for recovery of CMV, but in some cases, the virus has been isolated using only a throat swab (Dominguez et al., 1993; Visseren et al., 1997). It must be recognized that the recovery of CMV from urine should be interpreted only as evidence of infection, not necessarily as an etiologic agent of disease, with the exception of very early detection (0 to 14 days postnatal) in congenitally infected neonates. The resurgence of mumps in the United Kingdom and sporadic outbreaks in the United States since 2003 has prompted further development of rapid diagnostic techniques, since antimumps immunoglobulin M (IgM) may take 10 days to appear. Krause et al. showed that, for oral samples, real-time RT-PCR has superior sensitivity (98%) for mumps detection compared to both nested PCR (89%) and a combination of culture and antigen detection methods (83%) (Krause et al., 2006). Urine was an inferior specimen, however, for all three methods, RT-PCR (30%), nested PCR (2%), and combination culture and antigen detection (34%). The polyomaviruses, JC virus (JCV) and BKV, can be detected by immunologic methods in shell vial cell cultures, but detection by nucleic acid amplification is more sensitive than isolation of the virus in cell culture (Marshall et al., 1990).

In the immunocompetent host, HSV (corneal infections) and adenoviruses (conjunctivitis) are the most common ocular viral pathogens. Detection of either virus is best determined from a conjunctival swab or corneal scrapings by molecular testing or culture. Immunosuppressed individuals in general, and HIV-infected individuals in particular, are most at risk of retinitis due to CMV, and molecular testing can provide sensitive and specific diagnostics for CMV retinitis. For example, Yamamoto et al. determined that, in a group of AIDS patients with retinitis, 88.1% of aqueous humor specimens from eyes with active retinitis were positive for CMV DNA and 78.4% of those specimens from ocular sites became CMV DNA negative after treatment (Yamamoto et al., 2003).

Generally, lung and other tissue from the respiratory tract and brain tissue are the only specimens from organs that yield viruses in cell cultures. Occasionally, liver and, rarely, spleen tissue have yielded CMV or HSV. Molecular testing has extended the utility of fresh and formalin-fixed, paraffin-embedded (FFPE) tissue, allowing the detection of various viral nucleic acids. Fresh tissue is simply digested, and then the lysate is used for nucleic acid extraction. FFPE-preserved tissue undergoes several washings in xylene to remove the paraffin and then in alcohol to remove the xylene. Then the sample is subsequently digested with a detergent and a protease to make a lysate that is compatible with nucleic acid extraction procedures. This process has been used with varying levels of success, and some of the data come from molecular genetics studies. Giannella et al. found that the best fixative for recovery of nucleic acids for breast tissue was ethanol and then Histochoice, and formalin was the worst (Giannella et al., 1997). A study of gastric carcinoma showed that the time the tissue spent in fixative was of vital importance. Positive results were found 100% of the time for tissue fixed for 1 day (6 of 6). After 2 to 3 days of fixation, only 7 of 16 were positive, 4 to 6 days of fixation showed 4 of 28 positive, and no samples were positive after more than 7 days in formalin (Inoue et al., 1996). The variables seem to indicate that better detection is correlated with shorter formalin fixation times or use of nonformalin fixatives. Similarly, a study that sequenced a segment of JCV DNA from kidney tissue after a year in frozen storage demonstrated that the best sequence came from fresh-frozen tissue. The next best source was FFPE-frozen tissue, which had lower quality, though acceptable, results. Formalin-soaked tissue, however, showed the most base substitutions in the sequence. Viral RNA can also be recovered from tissue, as shown by detection of WNV RNA from FFPE tissues where RT-PCR was more sensitive (89%) than immunohistochemistry (52%) (Bhatnagar et al., 2007).

TRANSPORT AND STORAGE

Swabs

The sensitivity of a test system, cell culture or molecular based, depends on the collective efficiency of all steps in acquiring a specimen from the patient to reporting the results to the clinical service. Recently, great improvements have been achieved in the orientation and preparation of the fiber material on the shaft of flocked swabs. Compared with previous methods of wrapping long strands of material

on the end of the swab shaft, short nylon fiber strands, which are electrostatically charged, are bonded at right angles to the surface of the swab. The product (Copan, Inc., Corona, CA) has resulted in more efficient collection and release of particulate matter from flocked swabs (Chernesky et al., 2006). The flocked swabs have enhanced the ability to detect nucleic acid targets in several bacteria and reduced the time required to detect several viruses in cell cultures (Chernesky et al., 2006; Drake et al., 2005; Dunn et al., 2003). In addition, the flocked swabs were more efficient for the collection of epithelial cells from the upper respiratory tract than traditional wrapped swabs (Daley et al., 2006).

Specimen Transport

In general, viruses that are enveloped, such as the herpesviruses, HIV, and the myxo- and paramyxoviruses (especially RSV), are relatively labile compared to those without envelopes (Tjotta et al., 1991). Similarly, survival of viruses is favored by cool temperatures (4°C) and an approximately neutral pH (Abad et al., 1994). Nevertheless, these viruses survive transit for at least 1 to 3 days if maintained at 4°C (Shinkai et al., 1997). HSV can survive for as long as 2 h on the surface of skin, 3 h on cloth, and 4 h on plastic (Turner et al., 1982). Molecular testing, however, abrogated the need for viral viability in the quest for sensitive detection. This is a distinct advantage, since an unexpected delay in sample processing due to an error in communication, handling, or the weather will affect the quality of the test and result for the patient minimally. This is unlike viral culture, where each hour of delay before inoculation translates to progressively lower probability of successful isolation. At Mayo, we recommend that samples be refrigerated (2 to 8°C) for all tests. Ambient transport is acceptable for JCV and BKV assays if the sample reaches the lab within 24 h. Frozen specimens are also acceptable for most tests, except when testing for CMV. After the result is reported, patient specimens are stored for 21 days in the laboratory at 2 to 8°C.

Specimen Storage

Viruses such as adeno- and enteroviruses that do not have structurally labile lipid envelopes survive freeze-thaw procedures with relatively little loss of viral titer. Conversely, a single freeze-thaw cycle may decrease the titer of HSV by 100-fold in cell culture. Even storage at room temperature for 1 to 30 days significantly reduced the infectivity of HSV. For short-term (<30 days) transit or storage of most viral suspensions, the specimen should be held at 2 to 8°C rather than frozen. In distinct contrast, target nucleic acids can be detected by molecular methods for months (Jerome et al., 2002). In this study design, swabs from a variety of anatomical sites were placed in a PCR medium, and some of the raw sample was frozen, while some of the specimen was extracted for quantitative real-time PCR testing. The raw samples were stored at −20°C, and the nucleic acid extracts were stored at 4°C for 16 months. The raw samples were then thawed, extracted, and tested alongside the stored extracts. Extracts stored for 16 months demonstrated quantitative results that showed a 0.987 correlation using regression analysis with the initial results, with 86% agreement within 0.5 log and 93% agreement within 1 log of the initial quantitative result. Raw samples stored for 16 months and then extracted and tested showed a correlation slope of 0.996, with 76% agreement within 0.5 log and 94% agreement within 1 log, compared to the initial results. Finally, the results of tests for stored extract were compared to results of tests for extracts of the stored raw specimens. This comparison showed a 0.998 slope of correlation, with 86% agreement within 0.5 log and 97% within 1 log. These results clearly demonstrate that freezing patient specimens and refrigerating nucleic acid extracts of specimens are both reasonable for at least 16 months. This holds true for HSV type 2, as tested, but may be indicative for the other herpesviruses, and could also hold true for DNA viruses in general. Another group showed that quantification of concentrations of nucleic acids from HBV (DNA) and HCV (RNA), remained stable in patient samples after 8 freeze-thaw cycles as determined by a commercial detection platform (Krajden et al., 1999). An alternative approach was taken for storage of respiratory swab specimens in a study by Krafft et al. (Krafft et al., 2005). Swabs were stored in ethanol at ambient temperatures for 1 month or 6 months, and the results of molecular testing (for influenza viruses A and B and adenovirus) were compared to culture results at the time of specimen collection. The results of RT-PCR tests on the stored specimens demonstrated a high correlation to initial culture results and even detected culture-negative, molecular test-positive specimens. In another study, RT-PCR was 82% sensitive (and more sensitive than repeat culture) when used to detect influenza virus in nasal aspirates stored frozen for 1 to 3 years at −70°C compared to viral culture at the time the aspirate was taken (Frisbie et al., 2004). These are important studies with consequences for daily laboratory practices, and more like them are needed.

FUTURE CONSIDERATIONS

Major diagnostic methods for detection of viral infections have been cell culture (conventional tube and shell vial), fluorescent-antibody direct staining of specimen smears on slides, antigen detection by EIA, and amplification and detection of target nucleic acids by PCR. During the last 15 to 20 years, we have experienced breakthrough changes in technology for the rapid, sensitive, and specific diagnosis of viral infections by molecular amplification methods (Espy et al., 2006; Smith and Cockerill, 2002). Initially, laboratories implemented qualitative and quantitative molecular technology methods for the diagnosis and monitoring of HIV and the HBV and HCV infections. Later, routine molecular tests became commercially available for detection of HPV, HSV, VZV, CMV, and EBV infections. In addition, home brew PCR assays have been developed for diagnostic purposes for enteroviruses, polyomaviruses (JCV and BKV), influenza virus A and B, parvovirus, etc. (Smith and Cockerill, 2002). Finally, multiplex PCR and microarray systems have been developed for detecting a comprehensive list of viruses from the respiratory tract.

In addition to rapid detection of target viral nucleic acids, criteria for specimen selection are now becoming available (Hanson et al., 2007; Tang et al., 1999). As can be seen from Table 2, specimen selection for molecular testing is similar to culture but in many cases expands the choice of potential specimen sources.

The need for conventional tube cell culture detection of viruses and fastidious bacteria will likely be phased out in the coming years. FDA-approved molecular testing for *Chlamydia trachomatis* (an intracellular pathogen) has replaced cell cultures for the diagnosis of this bacterium from a variety of specimen types (Jespersen et al., 2005). Collectively, our diagnostic virology laboratory has converted 95% of our cell culture assays to molecular methods (Table 1).

The last 10 years have been particularly eventful. Real-time closed PCR platforms have permitted PCR technology to be incorporated into the laboratory. Importantly, integration of rapid and sensitive PCR tests have facilitated result reporting, usually during the same day the specimen was submitted to the laboratory (Zitterkopf et al., 2006). The proliferation of nucleic acid testing will continue to impact specimen sampling, storage, and processing in the coming years.

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Primary Isolation of Viruses

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3

Viruses are obligate intracellular parasites and, therefore, require living cells in which to replicate. The living cells essential for virus isolation and assay can be in the form of cultured cells, embryonated eggs, or laboratory animals, such as newborn mice. Since the optimum growth conditions for different viruses differ tremendously, a great variety of methods and host systems need to be employed to isolate the broadest spectrum of viruses from clinical specimens. Culture has long been considered the gold standard for diagnosis. It is more open-minded than other methods, and the unexpected or unknown agent can be recovered. Thus, culture methods continue to play an important role in the discovery of new viruses (van den Hoogen et al., 2001; Ksiazek et al., 2003; Fouchier et al., 2004; van der Hoek et al., 2004).

In current practice, specialized cell culture systems, embryonated eggs, and laboratory animals are confined to research or major public health reference laboratories. Cell cultures in monolayers are the sole isolation system utilized in routine diagnostic laboratories. The past decade has witnessed conventional cell culture methods being supplemented or even replaced by more rapid and targeted cell culture methods. These newer methods can be performed by less experienced personnel, with less labor, and with results reported within 1 to 2 days of culture inoculation. Despite the increasing availability of viral antigen and nucleic acid tests, culture methods remain important, especially in the hospital setting. This chapter will focus on those isolation methods currently used in routine clinical laboratories.

VIRUS ISOLATION IN CELL CULTURES

The discovery by Enders, Weller, and Robbins in the late 1940s that poliovirus replicates in cultivated cells derived from non-nervous system mammalian tissues revolutionized and simplified procedures for the isolation of viruses (Enders et al., 1949). Until that time, intact animals and embryonated eggs were the common systems used. After that landmark discovery, cell cultures were prepared for virus studies from a wide variety of animal and human tissues, and many of the common viruses we are familiar with today were discovered. In the 1980s, the introduction of shell vial centrifugation cultures, with antibody staining at 1 to 2 days to detect virus infection, constituted a major advance. The development of suspension cultures of human lymphocytes allowed the discovery and cultivation of human retroviruses (Barre-Sinoussi et al., 1983; Derse et al., 2004) and several human herpesviruses (Black et al., 1989; Yao et al., 1985).

Types of Cell Culture

Cell cultures are generally separated into three types (Table 1): primary cells, which are prepared directly from animal or human tissues and can usually be subcultured for only one or two passages; diploid cell cultures, which are usually derived from human tissues, either fetal or newborn, and can be subcultured 20 to 50 times before senescence; and continuous cell lines, which can be established from human or animal tissues, from tumors, or following the spontaneous transformation of normal tissues. These have a heteroploid karyotype and can be subcultured an indefinite number of times. However, sensitivity to virus infection may change after serial passage and after passage in different laboratories.

Recent innovations in monolayer cell culture include the use of mixtures of two or three different cell lines and genetically altered cells to enhance sensitivity or facilitate detection of virus infection.

Variation in Sensitivity to Different Viruses

Cell cultures vary greatly in their sensitivity to different viruses (Table 2). If a virus is inoculated into an insensitive cell culture, the virus will not be able to replicate, and a negative result will be obtained. When small amounts of virus are present in a clinical sample, a positive result may be obtained only when the most sensitive systems are used. Therefore, it is important that those caring for the patients inform the laboratory of the clinical syndrome and/or virus(es) suspected, so that the most sensitive cell cultures can be used and appropriate detection methods employed. Laboratories should periodically monitor the sensitivity characteristics of cell cultures, since significant changes can occur over time (Landry et al., 1982; Coffin and Hodinka, 1995; McCarter and Robinson, 1997).

Supplies and Equipment Needed

The materials needed for the isolation of viruses in cell culture (Table 3) are those necessary for the safe handling and inoculation of cell cultures, maintenance and observation of cell cultures, and preservation and storage of clinical specimens and virus isolates. Although the clinical virologist is primarily interested in virus isolation, maintaining different cell cultures in a healthy condition is absolutely necessary to

TABLE 1 Types of cell cultures commonly used in a clinical virology laboratory

Cell culture[a]	Examples	No. of subpassages
Primary	Kidney tissues from monkeys, rabbits, etc.; embryos from chickens, guinea pigs, etc.	1 or 2
Diploid	Human embryonic lung (MCR-5) or human newborn foreskin	20–50
Continuous	Human epidermoid carcinoma of lung (A549), mink lung (ML or Mv1Lu)	Indefinite

[a]Mixtures of different cell cultures within a single tube or well are now commonly used. Some continuous cell lines have been genetically engineered to provide a reporter system for rapid and simplified detection or for greater sensitivity.

ensure good results. A wide variety of cell cultures are available commercially and can be purchased and delivered once or twice a week according to the needs of the laboratory. Both the quantity and types of cell cultures used will vary with the seasonal variations in virus activity. The use of cryopreserved cell cultures, which can be stored at –70°C and thawed for use as needed, can provide additional flexibility (Huang et al., 2002a; Leland and Ginocchio, 2007).

Obtaining and Processing Specimens

Although this area has been reviewed in the previous chapter, it should be reiterated that without appropriate specimens that are properly collected early in illness and promptly transported to the laboratory, the subsequent time and effort spent in isolation attempts will be wasted. Accomplishing this is an important task of the clinical virology laboratory and requires continuing communication with and education of the clinicians.

Conventional Cell Culture

Conventional cell cultures in clinical laboratories have traditionally grown as monolayers in screw-cap roller tubes, inoculated and incubated as described in the procedure below. However, cultures in 24-well plates and shell vials also can be used and observed for cytopathic effect (CPE). To isolate a spectrum of viruses in conventional culture, cells of several different types are inoculated, such as human diploid fibroblasts (HDFs), a human heteroploid cell line (e.g., A549), and a primary monkey kidney cell culture. Alternatively, limited cultures intended to detect only one or two virus types can be performed. The cell type(s) most sensitive to the suspected viruses in the clinical specimen should be included. Ideally, only healthy, freshly prepared, young cell cultures should be used because aged cells are less sensitive to virus infection. All cell cultures should be examined under the microscope before inoculation to ensure that the cells are in good condition.

TABLE 2 Variation in sensitivity of conventional cell cultures to infection by viruses commonly isolated in a clinical virology laboratory

Virus	Sensitivity in cell culture[a]:			
	PMK	HDF	HEp-2	A549
RNA viruses				
Enterovirus	+++	++	+/–	+/–
Influenza virus	+++	+	–	–
Parainfluenza virus	+++	+	+/–	+/–
RSV	++	+	+++	+/–
Rhinovirus	+	+++	+	–
DNA viruses				
Adenovirus	+	++	+++	+++
CMV	–	+++	–	–
HSV	+	++	++	+++
VZV	+	+++	–	+++

[a]PMK, primary MK. Degree of sensitivity: +++, highly sensitive; ++, moderately sensitive; +, low sensitivity; +/–, variable; –, not sensitive.

TABLE 3 Supplies and equipment needed for isolation of viruses in cell culture

Procedure	Supplies and equipment needed
Inoculation of cell cultures	Laminar flow hood, centrifuge, pipettes, automatic pipetting device, pipette jar and discard can, disposable gloves, disinfectant, and sterile glass- and plasticware
Maintenance of cell cultures	Culture media, serum, antibiotics, 4°C refrigerator, test tube racks and/or rotating drum, shell vial racks, room air incubators, CO_2 incubator, water bath, and upright and inverted microscopes
Staining of shell vials and identification of virus isolates	Centrifuge, centrifuge tubes, PBS, Teflon-coated microscope slides, forceps, incubator, monoclonal antibodies and reagents, mounting medium, fluorescence microscope
Preservation and storage of viruses	Freezer vials, ultra-low-temperature freezer (–70°C), and dimethyl sulfoxide as stabilizer

Inoculation and Incubation

Although techniques may vary somewhat for different viruses, in general, the following procedures apply for noncentrifuged conventional cultures:

1. Pour off or aspirate culture media and inoculate specimens (0.1 to 0.3 ml) into each culture tube. Uninoculated cultures should be kept in parallel for comparison.
2. Allow specimen to adsorb in the incubator at 35 to 37°C for 30 to 60 min. Then, add 1.0 to 1.5 ml of maintenance medium and return to the incubator. Place inoculated cultures in roller tubes in a rotating drum if available. Rotation is optimal for the isolation of respiratory viruses, especially rhinoviruses, and results in earlier appearance of CPE for many viruses. If stationary racks are used, it is critical that culture tubes be positioned so that the cell monolayer is bathed in nutrient medium, otherwise the cells will degenerate, especially at the edge of the monolayer.
3. Examine inoculated culture tubes daily for the first week and then every other day for virus-induced CPE.

Compare with uninoculated control tubes from the same lot of cell cultures.

4. Certain specimens, such as urine and stool, frequently will be toxic to the cell cultures and this toxicity can be confused with virus-induced CPE. With such specimens, it is a good practice to check inoculated tubes, either after adsorption or within 24 h of inoculation, and refeed with fresh medium if necessary. If toxic effects are extensive, it may be necessary to subpassage the inoculated cells to dilute toxic factors and provide viable cells for virus growth.

5. Bacterial or fungal contamination will require filtration, using a 450-μm-pore-size filter, of either the inoculated culture supernatant fluid or the original specimen, followed by inoculation of fresh cultures.

6. Inoculated cultures and the uninoculated controls are generally kept for observation for virus-induced effects for 10 to 14 days. Exceptions include cultures for herpes simplex virus (HSV) only, which may be terminated at 7 days, and for cytomegalovirus (CMV), which are commonly kept for 3 to 4 weeks. During this time, cell cultures may need to be refed to maintain the cells in good condition. Some cultures, such as HEp-2 cells, may require refeeding or subculturing every few days. Great care must be taken when refeeding cultures, so cross-contamination from one specimen to another does not occur. Separate pipettes should be used for separate specimens. Aerosols, spatter, and contamination of test tube caps and gloves should be avoided.

7. When virus-induced effects occur and progress to include 25 to 50% of the monolayer, specific identification can usually be obtained by immunofluorescence (IF) of infected cells. Passage of infected cultures, especially in doubtful cases, into a fresh culture of the same cell type may be necessary to ensure recovery of virus for further identification of the isolate. For certain cell-associated viruses, such as CMV or varicella-zoster virus (VZV), it is necessary to trypsinize and passage intact infected cells. Adenovirus can be subcultured after freezing and thawing infected cells, which disrupts the cells and releases intracellular virus.

8. For certain fastidious viruses, when the amount of infectious virus in the specimen is low or when the patient has received antiviral therapy, blind passage (i.e., subculture of the inoculated culture in the absence of virus-induced effects) into a set of fresh culture tubes may be necessary before virus growth can be detected.

Detection of Virus-Induced Effects

CPE

Many viruses can be identified by the characteristic cellular changes they induce in susceptible cell cultures. These can be visualized under the light microscope. Examples of CPE characteristic for a number of common viruses are shown in Fig. 1 and described in greater detail in the sections on individual viruses. The degree of CPE is usually graded from + to ++++ as it progresses from involving less than 25% of the cell monolayer (+) to 50% (++), 75% (+++), and finally, 100% (++++). There are two important points that should be emphasized regarding CPE induced by virus:

1. The rate at which the CPE progresses may help to distinguish similar viruses. For example, HSV progresses rapidly to involve the entire monolayer of several cell systems (Fig. 2), whereas two other herpesviruses, CMV and VZV, grow primarily in HDFs and progress slowly over a number of days or weeks.

2. The type of cell culture(s) in which the virus replicates is important. Although the CPE may be similar within a virus group, the susceptibility of different cell types to different viruses may differ greatly. For example, both poliovirus and echovirus induce similar CPE in primary rhesus monkey kidney (RhMK) cells; however, echovirus does not induce CPE in HEp-2 cells, thus allowing presumptive identification (Fig. 3).

Virus-induced CPE must be distinguished from nonspecific CPE caused by toxicity of specimens, contamination with bacteria or fungi, or old cells. A subculture onto fresh cells should amplify virus effects and dilute toxic effects. On occasion, foci of cells inoculated onto the culture monolayer from the original specimen or from another cell culture can be mistaken for viral CPE. With experience, the appearance of the cellular changes, taken together with the susceptible cell systems, the specimen source, and clinical disease, usually allow a presumptive diagnosis to be made as soon as the virus-induced cellular changes occur.

Hemadsorption

Parainfluenza and, sometimes, influenza viruses may not induce distinctive cellular changes; however, these viruses possess hemagglutinins, which have an affinity for red blood cells (RBC) and are expressed on the surface of infected cells. When a freshly obtained guinea pig RBC suspension is added to the infected cultures, the RBC adsorb onto the infected cells, resulting in a hemadsorption phenomenon, as shown in Fig. 4B. When aged guinea pig RBC are used, however, nonspecific hemadsorption can occur (Fig. 4C) and must be distinguished from that resulting from a specific viral infection. Furthermore, the hemadsorption test is performed at 4°C to 22°C because the RBC will elute when incubated at 37°C. When a culture shows positive hemadsorption, infected cells are transferred to a slide and stained by IF to identify the causative virus. Alternatively, the culture fluid can be subcultured into a fresh culture to confirm and amplify the virus isolation and to permit further identification.

It should be noted that not all viruses that agglutinate RBC can adsorb them onto infected cell monolayers. Rather, hemadsorption is a property of those viruses that bud from the host cell membrane during maturation and thus express viral hemagglutinin on the surface of the infected cell.

Blind Immunostaining

To more rapidly detect virus growth, intact cell culture monolayers can be fixed and stained with fluorescein- or horseradish peroxidase-labeled antibodies to viral antigens, usually 1 to 5 days after inoculation. Alternatively, at the end of the observation period, cells can be scraped or trypsinized from roller tubes, affixed to glass slides, and blindly stained for detection of viral antigens prior to discard.

Identification of Virus Isolates

Presumptive identification of virus isolates often can be made on the basis of characteristic virus-induced effects (e.g., type of CPE or hemadsorption) and selective cell sensitivity. Final identification is most commonly made by staining infected cells with fluorescein-labeled antibodies. Monolayers showing CPE or hemadsorption are trypsinized or dislodged, and the cells are transferred to a welled slide and then stained with specific antibody. In more difficult cases, PCR also can be used for virus

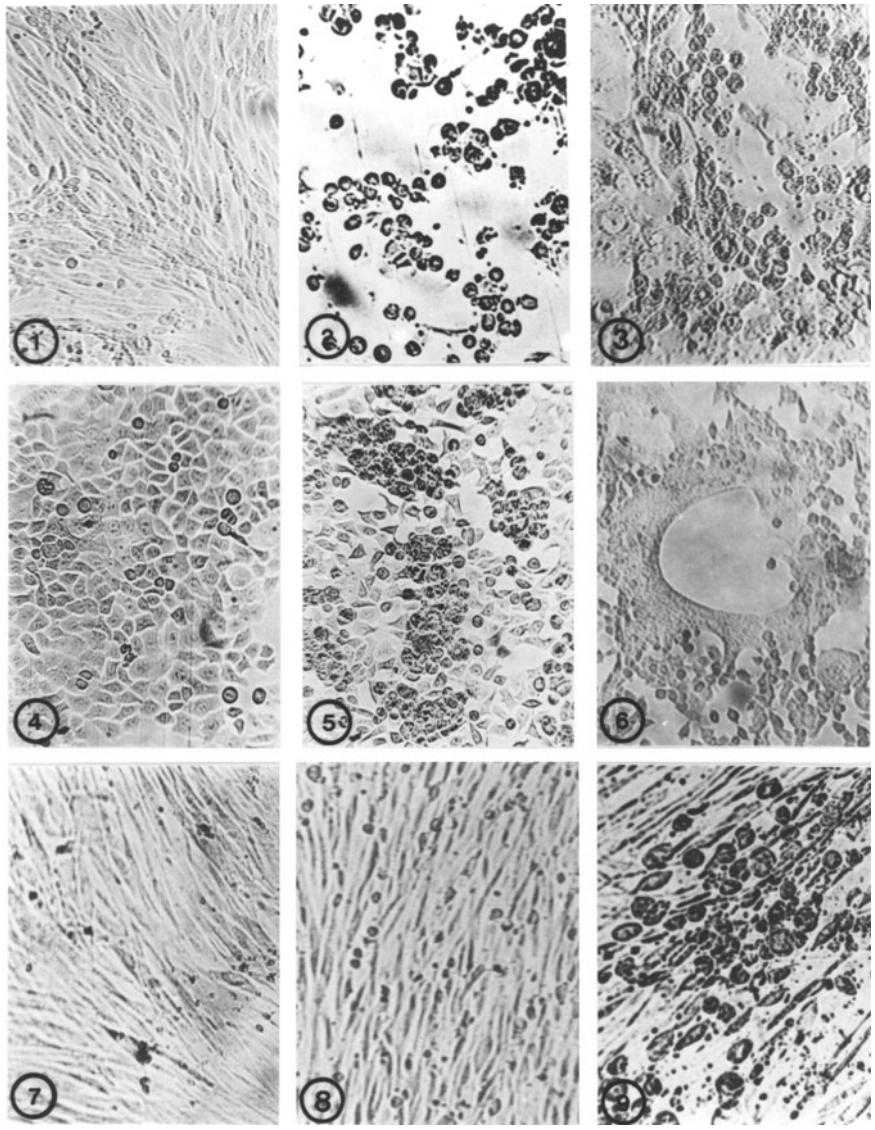

FIGURE 1 Examples of characteristic CPE of different viruses. (1) Uninfected RhMK cells; (2) poliovirus CPE in RhMK cells; (3) influenza B virus CPE in RhMK cells; (4) uninfected HEp-2 cells; (5) adenovirus CPE in HEp-2 cells; (6) RSV CPE in HEp-2 cells; (7) uninfected HDFs; (8) rhinovirus CPE in HDFs; (9) CMV CPE in HDFs.

identification. When determination of specific serotype is requested for enteroviruses or adenoviruses, neutralization of virus-induced cytopathology in cell culture can be performed. However, these traditional, labor-intensive methods are now rarely performed in clinical laboratories and are being replaced by PCR with sequencing (Oberste et al., 1999, 2005).

Occasionally a new isolate cannot be identified by the standard tests. The morphologic properties of the infecting virus can be determined by electron microscopy, if available, with subsequent identification by molecular techniques. Several new viruses recognized recently in this manner include human metapneumovirus isolated in tertiary monkey kidney cells (van den Hoogen, 2001), severe acute respiratory syndrome (SARS) coronavirus isolated in Vero E6 cells (Ksiazek et al., 2003), and NL-63 coronavirus isolated in tertiary monkey kidney cells (van der Hoek et al., 2004; Fouchier et al., 2004).

RAPID CULTURE METHODS

Centrifugation Culture (Shell Vial Technique)

The rapid diagnosis of viral infections is important in patient management. One of the most significant contributions to rapid diagnosis in the clinical laboratory has been the application of centrifugation cultures to viral diagnosis.

For a number of years, it has been recognized that low-speed centrifugation of monolayers enhances infectivity of viruses (Hudson et al., 1976) as well as chlamydia. The

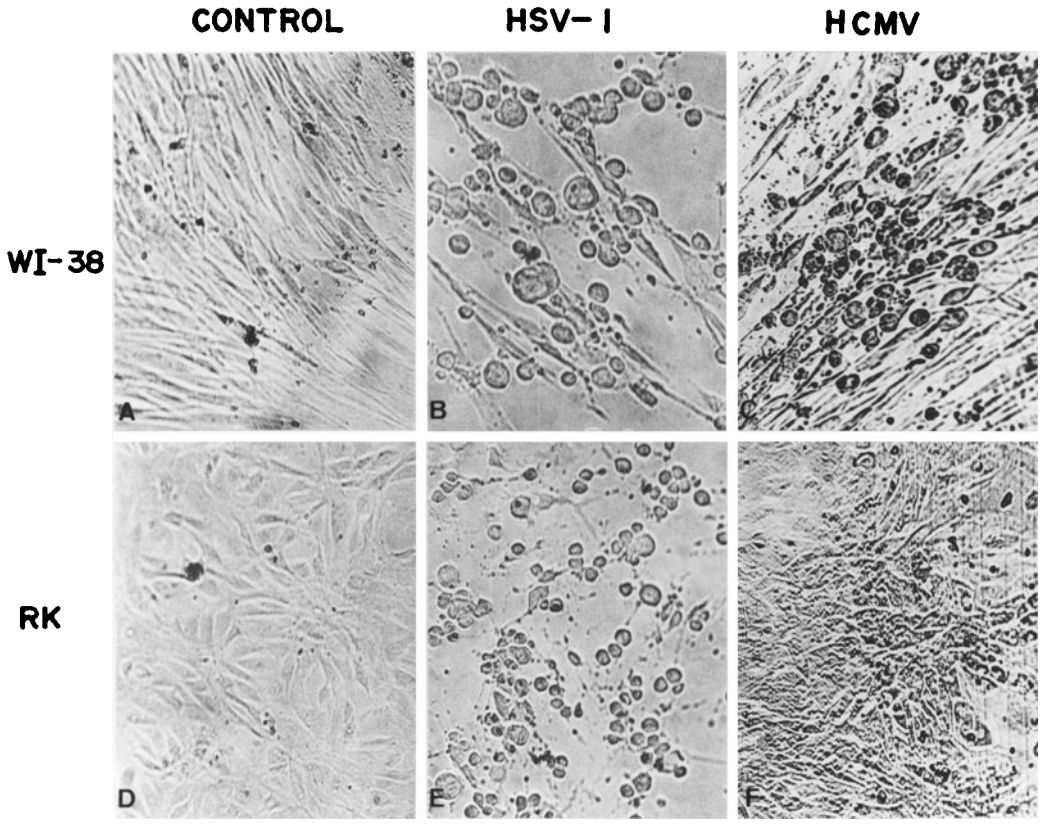

FIGURE 2 Cell sensitivity and rate of progression of CPE of two herpesviruses: HSV-1 and human CMV (HCMV). (A) Uninfected WI-38 cells; (B) extensive HSV-1 CPE in WI-38 cells, 2 days postinoculation; (C) CMV in WI-38 cells, 1 week postinoculation; (D) uninfected RK cells; (E) extensive HSV-1 CPE in RK cells, 1 day postinoculation; (F) absence of CMV CPE in RK cells, 2 weeks postinoculation. (Modified from Hsiung et al., 1994.)

mechanism for this effect is unclear and may involve centrifugation of virus aggregates or of virus attached to cell debris or an effect on cell membranes to enhance virus entry.

In 1984, the use of centrifugation cultures followed by staining with a monoclonal antibody at 24 h postinoculation was first reported for CMV (Gleaves et al., 1984) (Color Plate 1). Subsequent reports documented its usefulness in rapid diagnosis of other viruses, including HSV, VZV, adenovirus, respiratory viruses, and polyomavirus BK (Gleaves et al., 1985, 1988; Espy et al., 1987; Marshall et al., 1990; Olsen et al., 1993). In addition, when the inoculum is standardized, semiquantitative results can be obtained by counting the number of virus-positive cells (Slavin et al., 1992).

The shell vial technique usually combines (i) cell culture to amplify virus in the specimen, (ii) centrifugation to enhance viral infectivity, and (iii) early detection of virus-induced antigen (before CPE) by the use of high-quality specific antibodies. It can be used for any virus that replicates in cell culture and for which a specific antibody is available. For viruses with a long replication cycle, such as CMV, viral antigens produced early in the replication cycle can be detected many days before CPE is apparent by light microscopy. For viruses that replicate faster, e.g., HSV, or if the antibodies available are directed toward late rather than early replication products, less time is gained for detecting positives using the shell vial technique (Table 4). However, shell vial cultures can be terminated and negatives reported at 2 days rather than at 7 to 14 days.

It should be noted that centrifugation cultures can also be employed without early IF staining, but rather can be monitored for CPE and hemadsorption similar to conventional cultures in roller tubes. Centrifugation cultures can also be performed using 24- or 48-well tissue culture plates, instead of in shell vials.

The overall sensitivity of the shell vial technique varies with the type of specimen (Paya et al., 1987), the length and temperature of centrifugation (Shuster et al., 1985), the virus, the cell cultures, the antibody employed, and the time of fixation and staining. The use of young cell monolayers (Fedorko et al., 1989) with inoculation of multiple shell vials enhances the recovery rate (Paya et al., 1988). Toxicity, particularly problematic with blood and urine specimens, can lead to cell death and the loss of the monolayer, necessitating blind passage of the specimen or specimen reinoculation.

Furthermore, with all rapid techniques that target specific viruses, only the virus sought will be detected. Conventional isolation using a spectrum of cell cultures can detect a variety of virus types, including unanticipated agents (Blanding et al., 1989). While maximal sensitivity and virus recovery is obtained by performing both conventional culture and centrifugation cultures in parallel (Rabella and Drew, 1990; Espy et al., 1991; Rabalais et al., 1992; Olsen et al., 1993),

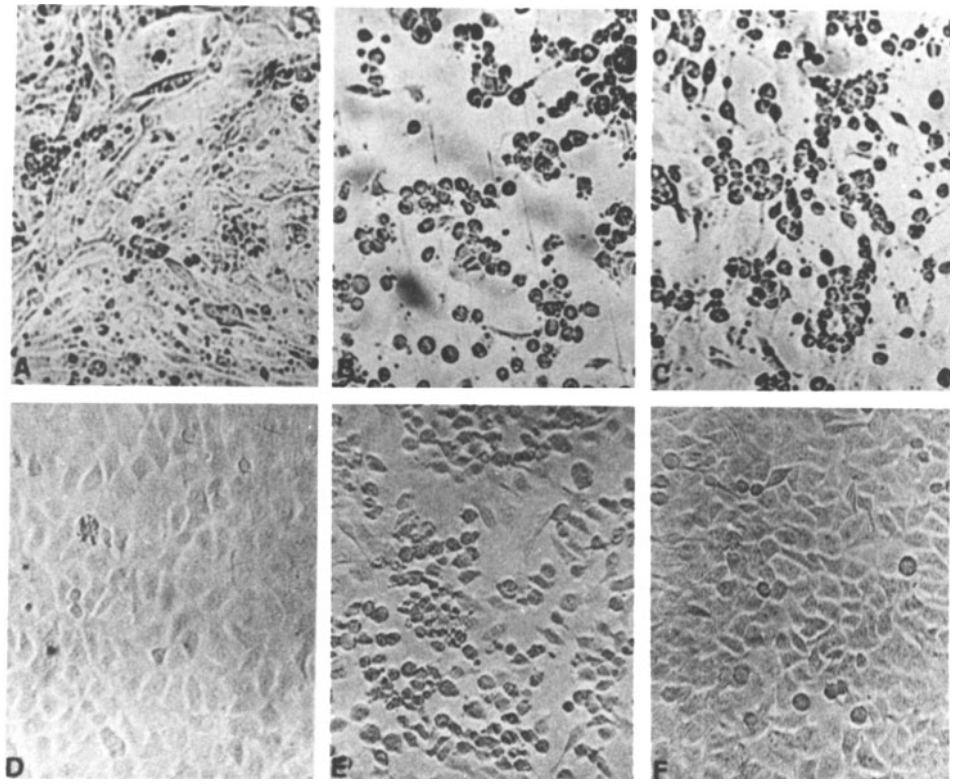

FIGURE 3 Differential sensitivity of cell cultures to enteroviruses. (A) Uninfected RhMK cells; (B) poliovirus-infected RhMK cells showing advanced CPE; (C) echovirus-infected RhMK cells showing advanced CPE; (D) uninfected HEp-2 cells; (E) poliovirus-infected HEp-2 cells showing advanced CPE; (F) echovirus-infected HEp-2 cells showing absence of CPE. (Reprinted from Hsiung et al., 1994, with permission of the publisher.)

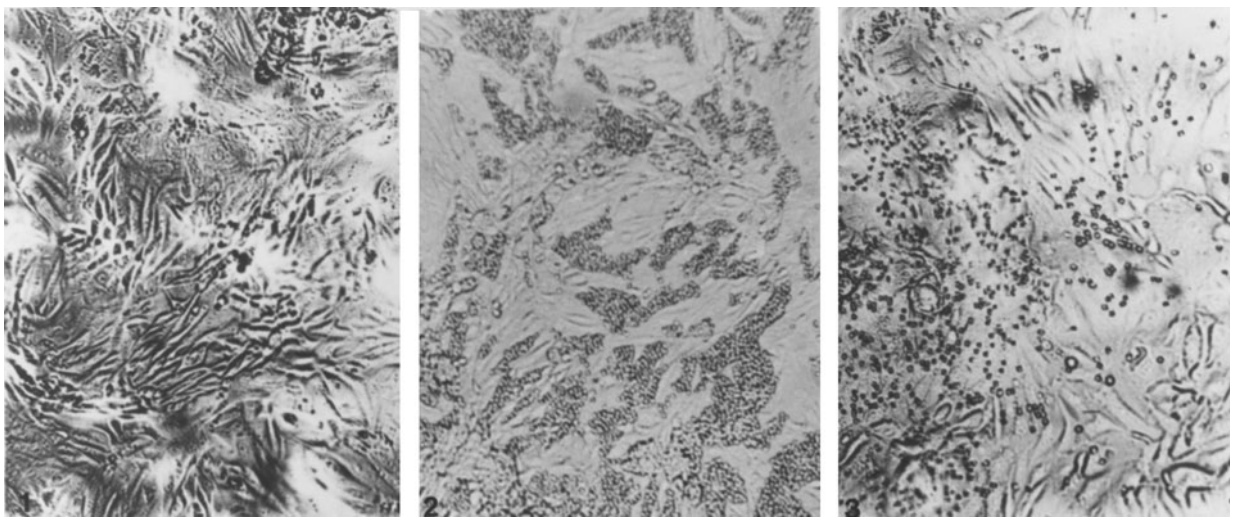

FIGURE 4 Hemadsorption of guinea pig RBC by parainfluenza virus in MK cells. (A) Uninfected MK cells; (B) specific hemadsorption in parainfluenza virus-infected MK cells; (C) nonspecific hemadsorption seen with aged RBC in uninfected cell cultures. (Modified from Hsiung et al., 1994.)

TABLE 4 Time to virus detection by conventional and centrifugation culture[a]

Virus	Time (days) to virus detection	
	Conventional culture [avg (range)]	Shell vial centrifugation culture[b]
Respiratory viruses		
Adenovirus	6 (1–14)	2–5
Influenza A virus	2 (1–7)	1–2
Influenza B virus	2 (1–7)	1–2
Parainfluenza virus 1–4	6 (1–14)	1–2
RSV	6 (2–14)	1–2
Herpesviruses[c]		
CMV	8 (1–28)	1–2
HSV	2 (1–7)	1–2
VZV	6 (3–14)	2–5

[a]Modified from Landry, 2006.
[b]Incubation time prior to fixation and immunostaining with monoclonal antibodies.
[c]Isolation of Epstein-Barr virus and HHV-6 requires cocultivation with umbilical cord lymphocytes. Routine diagnosis is by antibody response.

to conserve resources, this combined approach might be limited to selected patient groups and sample types.

Reagents and Equipment

Antibodies to specific viral types, usually fluorescein or rhodamine labeled
Cold acetone
Shell vials; 1 dram, 15 by 45 mm, with caps or stoppers
Coverslips; 12-mm diameter
Cell cultures grown on coverslips in shell vials, sensitive to the suspected viruses
Low-speed centrifuge with adapters for shell vials
Humidified chamber
Rotator or rocker
Suction flask and vacuum source

Test Procedure

Inoculation of Shell Vials (Traditional Single-Cell Culture Type, Stained for One Virus)

1. Prepare 2 shell vials.
2. Remove cap and aspirate medium from shell vial.
3. Inoculate prepared specimen onto monolayer, 0.2 to 0.3 ml per vial.
4. Replace cap and centrifuge (30 to 60 min at 700 × *g*).
5. Aspirate inoculum for blood, urine, and stool samples and then rinse with 1 ml of medium to reduce toxicity.
6. Add 1.0 ml of maintenance medium to each shell vial and incubate at 35°C for 1 to 2 days.

Fixation of Coverslips in Shell Vials

1. Before fixation, inspect the coverslips for toxicity, contamination, etc. If necessary, passage the cell suspension to a new vial and repeat incubation before staining.
2. If monolayer is intact, aspirate medium from shell vials and rinse once with 1.0 ml of phosphate-buffered saline (PBS) (pH 7). If monolayer appears fragile, do not rinse with PBS.
3. Aspirate completely, then add 1.0 ml of 100% cold acetone or 50/50 acetone/methanol to each shell vial and allow cells to fix for 10 min.
4. Aspirate the acetone and allow the coverslips in the shell vial to dry completely.

Staining of Coverslips

1. Add 1.0 ml of PBS to each coverslip and then aspirate the PBS.
2. Pipette 150 μl (5 drops) of antibody reagent (appropriately titrated and diluted) into the shell vial. Replace the cap.
3. Rock the tray holding shell vials to distribute the reagent and then check to see that no coverslips are floating above the reagent.
4. Place rack holding the shell vials in a humidified chamber in the 35°C incubator.
5. Incubate for 30 min.
6. Add 1.0 ml of PBS to the shell vial and then aspirate. Repeat wash step two additional times.

For direct assays (primary antibody is labeled), go directly to step 9. For indirect assays (primary antibody is not labeled), continue with step 7.

7. Pipette 150 μl (5 drops) of labeled conjugate onto the monolayer.
8. Repeat steps 3 to 6, except do not aspirate the last 1.0 ml of PBS.
9. Using forceps and a wire probe, remove coverslip and blot on tissue or absorbent paper, e.g., a Kimwipe.
10. Add 1 drop of mounting fluid to a properly labeled slide and place coverslip on mounting fluid with cell side down, being careful not to trap air bubbles.

Reading Procedure

Coverslips are examined using a 20× objective with a fluorescence microscope equipped with the appropriate filters to maximize detection of the fluorescein isothiocyanate label (or a light microscope if a peroxidase label is used). A known positive control is run for each viral antigen with each assay. Noninfected monolayers are fixed and stained as negative antigen controls. For indirect IF, normal goat serum or PBS plus fluorescein isothiocyanate conjugate is used as a negative serum control.

The pattern of fluorescence varies depending upon the virus sought, the antibody used, the cell culture, and the

stage of virus replication. Even a single cell, characteristically stained, is considered a positive result. The test should be repeated if (i) the staining pattern is not typical for the virus sought, (ii) nonspecific staining is observed, or (iii) the staining color is more yellow than green.

Mixed-Cell Cultures and Monoclonal Antibody Pools

The great success of rapid shell vial cultures for detection of individual viruses led to an impetus to simplify the process. Thus, antibodies to more than one virus, often with two or three different fluorescent labels, have been pooled (Brumback et al., 1993; Olsen et al., 1993; Engler and Pruess, 1997), and two to three different cell cultures have been combined in one vial (Brumback and Wade, 1994; Navarro-Mari et al., 1999; Huang and Turchek, 2000). This concept has been embraced by a commercial supplier, and the cultures have been further enhanced through genetic engineering (Table 5). Numerous reports have shown the value of these mixed-cell cultures. Some laboratories have eliminated conventional cell culture tubes and converted to shell vials with mixed cells (D. Stevenson, J. Hoffman, K. W. Beckman, and E. Matthews, 23rd Annu. Clin. Virol. Symp., poster M-59, 2007). When combined with IF staining using antibody pools, detection of common respiratory viruses is simplified, labor is reduced, results are more rapidly reported on both positives and negatives, and the need for primary monkey kidney cells is eliminated. Learning to read and interpret IF staining of shell vial cultures is much easier than reading direct immunofluorescence on clinical samples or conventional CPE. Also, mixed-cell cultures can be observed for CPE for 1 to 2 weeks if desired.

There are at present a variety of combinations of cultures to choose from, depending upon the viruses sought (Table 5). R-Mix (Mv1Lu and A549) and R-Mix Too (MDCK and A549) are commonly used with monoclonal antibody pools to rapidly detect selected respiratory viruses (Fong et al., 2000; Huang and Turchek, 2000; Barenfanger et al., 2001; Dunn et al., 2004; St. George et al., 2002; Fader, 2005; Weinberg et al., 2004). Other viruses can be detected by observing the cultures for CPE. R-Mix Too was developed to avoid inadvertently growing SARS coronavirus. In contrast to Mv1Lu cells, MDCK cells will not support the growth of SARS coronavirus. Human metapneumovirus also can be isolated and detected by IF (K. L. McRae, J. A. Bocchini, Jr., A. Anga, and J. M. Matthews-Greer, 23rd Annu. Clin. Virol. Symp., poster TP-45, 2007).

The original E-Mix A (RD and H292) and E-Mix B (BGMK and A549) cells provided an advantage over the 3 to 5 conventional cell cultures traditionally inoculated to detect enteroviruses. However, E-Mix A and B cells were discontinued and replaced with the more sensitive, genetically engineered Super E-Mix described below (Buck et al., 2002).

H&V Mix (CV-1 and MRC-5) was developed for isolation of HSV type 1 (HSV-1), HSV-2, and VZV and can also detect CMV. Although all of these viruses can be detected after 1 or 2 days of incubation via IF staining, VZV requires staining at 2 and 5 days for optimal detection. Many other viruses can also replicate and be detected by CPE (Huang et al., 2002b).

The protocols for inoculation, incubation, and staining for commercially obtained mixed-cell cultures are generally those recommended by the supplier, modified as needed by the user (see procedure below). In general, 2 to 3 shell vials are inoculated. For respiratory viruses, one shell vial is stained with the antibody pool 1 day postinoculation (Color Plate 2A). If positive, a second shell vial is scraped and spotted onto an 8-well slide for identification by staining with individual antibodies (Color Plate 2B). If the day 1 screen is negative, a second shell vial can be scraped on day 2 of incubation and spotted onto both a single-well and an 8-well slide. If the screening reagent applied to the single-well slide is positive, the multiwell slide is then stained with individual antibodies. If the screening reagent is negative, the multiwell slide is discarded. A third shell vial can be observed for CPE for a longer period, since some slower-growing and low-titer viruses may be detected. In another approach, some laboratories may stain the second shell vial in situ and, if positive, use the third shell vial to prepare a multiwell slide.

For enteroviruses, two shell vials are needed and staining at 2 and 5 days is recommended. Samples that contain high titers of virus, such as stools, are generally positive by day 2, but for spinal fluids, up to 5 days is required (Buck et al., 2002).

Test Procedure

Inoculation of Mixed-Cell Shell Vials (Adapted from Diagnostic Hybrids, Athens, OH)

1. Determine the number of vials needed based on the staining protocol to be used: (i) one vial is required for each day the culture will be screened with the antibody reagent (i.e., for the respiratory viruses, staining is at 16 to 24 h and then again at 48 to 72 h, whereas for enteroviruses, staining is at 48 to 72 h and then at 5 to 7 days); (ii) one additional vial is required for preparing 8-well slides used to identify the viruses from positive respiratory virus screens or to monitor for CPE for other viruses if desired.
2. Examine monolayers prior to inoculation.
3. Remove cap and aspirate maintenance medium from shell vial.
4. Add 1.0 ml of appropriate refeed medium to each shell vial.
5. Inoculate 0.2 to 0.4 ml of prepared specimen onto each monolayer.
6. Replace cap and centrifuge (60 min at 700 × *g*).
7. Incubate at 35° to 37°C.

Fixation of Coverslips in Shell Vials

1. When a monolayer is ready to be stained using the appropriate reagent, remove the medium and add 1.0 ml of PBS.
2. Swirl to mix and then aspirate.
3. Repeat this wash with another 1.0 ml of PBS and then aspirate.
4. Add 1.0 ml of cold acetone and allow cells to fix for 5 to 10 min.
5. Aspirate acetone.

Staining of Coverslips

1. Add 0.5 ml of PBS to wet the monolayer; swirl and then aspirate completely.
2. Add 4 drops of the antibody reagent to the fixed monolayers of patient and control samples. Replace cap.
3. Rock to ensure complete coverage of the monolayer by the reagent.
4. Place shell vials in a 35° to 37°C incubator for 30 min.
5. Aspirate the reagent from the monolayer.
6. Add 1.0 ml of the 1× wash solution or PBS.
7. Aspirate the 1× wash solution or PBS. Repeat the wash step and aspirate.

TABLE 5 Newer cell culture methods used in clinical laboratories

Culture source	Cell culture composition	Targeted viruses	Principles	Reference(s)
In-house preparation	HEp-2, LLC-MK2, MDCK	RSV, influenza A and B viruses, parainfluenza virus 1, 2, and 3, adenovirus	Stain with MAb[a] pool at 48 h; if positive, scrape 2nd vial, make multiwell slide and identify pathogen by individual MAbs	Navarro-Mari et al., 1999
In-house preparation	MRC5 and A549	Adenovirus, CMV, HSV	Pool of antibodies raised in different species; second antibody with labeled anti-species, using 3 different labels	Brumback and Wade, 1994
R-Mix[b]	Mink lung (Mv1Lu) and A549	RSV, influenza A and B viruses, parainfluenza virus 1, 2, and 3, adenovirus; can detect MPV by IF; CPE for other viruses can be observed (HSV, CMV, enteroviruses)	Inoculate 3 shell vials for each specimen; stain with MAb pool at 24 h; if positive, scrape 2nd vial, make multiwell slide and identify pathogen by individual MAbs; if negative, scrape 2nd shell vial at 48 h, stain and make single-well and multiwell slides; stain single-well slide with pooled reagent; if positive, identify using multiwell slide and individual MAbs; 3rd vial can be observed for other CPE, or used for identification of positives on day 2	Barenfanger et al., 2001; Dunn et al., 2004; Fong et al., 2000; Fader, 2005; Huang and Turchek, 2000; St. George et al., 2002; Weinberg et al., 2004
R-Mix Too[b]	MDCK and A549	Same as R-mix, except not susceptible to SARS coronavirus	More sensitive than R-mix for respiratory viruses, especially adenovirus and influenza B virus; not susceptible to SARS coronavirus	
H&V Mix[b]	African green monkey kidney (strain CV-1) and MRC-5 cells	HSV-1 and -2, VZV; can also isolate CMV, mumps, measles, rotavirus, poliovirus type 1, simian virus 40, encephalitis viruses, rhinovirus, adenovirus, enteroviruses, RSV	Combination of cells highly sensitive to HSV and VZV; can observe for CPE and then confirm by IF or stain by IF before CPE develops; can stain shell vials for HSV at days 1 and 2 and for VZV at days 2 and 5.	Huang et al., 2002b
E-Mix A and E-Mix B (discontinued)	RD, H292 (E-mix A), BGMK, A-549 (E-mix B)	Enteroviruses	Inoculate shell vials and stain with MAb pool at 2 and 5 days; uses two shell vials instead of 3–5 cell lines	
Super E-Mix (discontinued)	BGMK-hDAF, CaCo-2	Enteroviruses	Inoculate 2 shell vials, stain with MAb pool at 2 and 5 days	Buck et al., 2002; Huang et al., 2002c
Super E-Mix[b]	BGMK-hDAF, A549	Enteroviruses	Same as above	
ELVIS[b]	BHK cell line with UL39 promoter and *Escherichia coli lacZ* gene	HSV-1 and -2	Positive cells stain blue with X-Gal; can type positive cultures by adding two type-specific MAbs	Crist et al., 2004; Kowalski et al., 2002; Patel et al., 1999; Proffitt and Schindler, 1995; Stabell et al., 1993; Turchek and Huang, 1999

[a]MAb, monoclonal antibody.
[b]Available from Diagnostic Hybrids, Inc.

For direct assays (primary antibodies are labeled), such as the respiratory virus direct IF screening kit, go directly to step 11. For indirect assays (primary antibodies are not labeled), such as the enterovirus indirect IF identification kit, continue with steps 8 through 10.

8. Add 4 drops of anti-mouse conjugate and rock to ensure coverage of the monolayer.
9. Place shell vials in a 35° to 37°C incubator for 30 min.
10. Repeat steps 5 through 7.
11. Add 1.0 ml of demineralized water and then aspirate.
12. Using forceps and a bent-tip needle on a syringe barrel or a wire probe, remove the coverslip and transfer it, monolayer-side down, to a small drop of mounting fluid on a properly labeled slide.

Reading Procedure

13. Examine the stained monolayers using a fluorescence microscope with magnifications between ×100 and ×400.
14. Examine positive and negative controls first. Be sure to examine the entire monolayer of cells. A positive reaction is one in which apple-green fluorescence is observed in infected cells.

Identification of Respiratory Virus Positives

If the result is positive for a respiratory virus, process a reserved replicate culture as a cell suspension and spot onto an 8-well Teflon-coated slide to identify which respiratory virus may be present and then proceed as follows.

(a) Add 1 drop of each individual virus direct IF reagent to its corresponding well on the 8-well specimen slide. Leave 1 well as a negative.

(b) For the respiratory virus antigen control slide, add 1 drop of each individual virus direct IF reagent to its corresponding labeled well. An antigen control slide should be stained only once, as it contains individual wells of virus-infected cells and noninfected cells.

Genetically Modified Cell Lines

Genetic modification of cell lines is an emerging technology with great potential for the diagnostic laboratory (Olivo, 1996). In the Super E-mix for enteroviruses described above, human decay-accelerating factor (hDAF) or CD55, a cellular receptor for several enteroviruses, was transfected into Buffalo green monkey kidney (BGMK) cells to enhance cell susceptibility to enterovirus isolation (Lublin and Atkinson, 1989; Huang et al., 2002c). BGMK-hDAF cells were then combined with the human colon adenocarcinoma cell line (CaCo-2) in a mixed-cell culture. The resulting Super E-mix cells in one culture vessel were reported to be more sensitive for enterovirus recovery than inoculation of three separate conventional tube cultures using primary rhesus monkey kidney, A549, and fetal foreskin (SF) cells (Buck et al., 2002). In the current Super E-mix, CaCo-2 cells have been replaced with A549 cells.

In another approach, genetic elements derived from viral, bacterial, or cellular sources can be stably introduced into a cell, and when the target virus enters the cell, an event in the viral replication cycle triggers the production of a measurable enzyme. In a simple histochemical assay, infected cells stain a characteristic color (Color Plate 3). This approach has been shown to be feasible for both DNA and RNA viruses (Stabell et al., 1993; Lutz et al., 2005), although different strategies are necessary for enzyme induction. In contrast to CPE, infected cells stained in the inducible system can be read by an untrained observer, and the earliest stages of infection can be reliably detected.

The acronym ELVIS (enzyme-linked inducible system) has been given to a commercially available cell system for isolation of HSV (Proffitt and Schindler, 1995). Transgenic baby hamster kidney (BHK) cells have been altered to include an HSV-specific promoter and an *Escherichia coli* LacZ reporter gene. The HSV-positive cells form a blue precipitate when reacted with a chromogenic substrate (5-bromo-4-chloro-3-indolyl-5-D-galactopyranoside [X-Gal]). Both positive and negative results can be reported in as little as 16 h. It is simple, sensitive, and rapid and can be used for the simultaneous detection, identification, and typing of HSV isolates from clinical specimens (Patel et al., 1999; Turchek and Huang, 1999; Kowalski et al., 2002; Crist et al., 2004). However, ELVIS remains somewhat less sensitive than the most sensitive of conventional cell culture systems when specimens contain only a few infectious HSV particles.

VIRUSES COMMONLY ISOLATED IN CELL CULTURE

HSV-1 and HSV-2

Vesicular fluids, throat swabs, and genital lesions are the most common sources for virus isolation. Both HSV-1 and HSV-2 infect a wide variety of cell cultures. Early studies demonstrated rabbit kidney (RK) and human embryonic kidney (HEK) cells to be very sensitive to HSV infection (Landry et al., 1982). Subsequent evaluations found MRC-5 to be more sensitive than RK cells from commercial suppliers (McCarter and Robinson, 1997). Four continuous cell lines, ML, RD, A549, and H292 cells, also are highly sensitive (Woods and Young, 1988; Johnston et al., 1990b; Hierholzer et al., 1993). Differences in sensitivity are more evident when specimens contain low titers of virus (Zhao et al., 1987), when collected late in illness, or transported a distance. Variations in susceptibility over time or between suppliers can be problematic. The recently introduced mixtures of two sensitive cell lines in one culture may help alleviate this problem.

HSV produces a rapid degeneration of cells, often appearing within 24 h of inoculation of the cell culture (Fig. 2). The CPE begins as clusters of enlarged, rounded, refractile cells and spreads to involve the entire monolayer, usually within 48 h. The formation of multinucleated giant cells also can be seen with HSV-2 and is more apparent in epithelial than in fibroblast cells. Subcultures are performed by passaging 0.2 ml of supernatant fluid to a fresh culture tube. Over 90% of positives will be identified within 3 to 5 days. Occasionally, CPE develops later when very low titers are present or if the patient is on antiviral therapy.

Centrifugation cultures in shell vials or 24-well plates can be stained from 1 to 3 days after inoculation; however, staining at 1 day may miss some low-titer samples (Espy et al., 1991).

Identification of virus as HSV and differentiation as HSV-1 or -2 is most readily done by IF using monoclonal antibodies (Miller and Howell, 1983: Balkovic and Hsiung, 1985). Genetically engineered cell lines (e.g., ELVIS) also can be used to isolate and identify HSV.

VZV

Vesicle fluid and lesion swabs are the usual sources for VZV isolation. VZV is difficult to grow, and prompt inoculation into cell culture is desirable.

HDF and A549 cells are the most sensitive cells for the isolation of VZV, although the virus also has been isolated in other human epithelial cells, primary MK cells, and CV-1 cells.

Cytopathology starts as foci of rounded, enlarged cells, as seen with HSV; however, the onset and progression are much slower and the foci of CPE tend to progress linearly along the axis of the cells, similar to CMV. However, VZV-infected foci degenerate more rapidly than those infected by CMV. CPE first appears 3 to 7 days after inoculation but may take 2 to 3 weeks. The virus is cell associated, and subpassages are performed by trypsinization and passage of infected intact cells to fresh monolayers of cells. Final identification is by IF using monoclonal antibodies.

Centrifugation cultures are significantly more sensitive than conventional roller tubes (Gleaves et al., 1988; Coffin and Hodinka, 1995). Staining of monolayers at 2 and again at 4 to 5 days after inoculation is recommended for optimal sensitivity. Use of mixed-cell cultures (Huang et al., 2002b) and antibody pools with different fluorescent labels may optimize detection of both HSV and VZV in a single culture (Brumback et al., 1993).

CMV

Virus can be isolated from a variety of specimens, including urine, saliva, tears, milk, semen, stools, vaginal or cervical secretions, peripheral blood leukocytes, bronchoalveolar lavage fluid, lung, liver, and gastrointestinal biopsy tissues.

HDFs are the single most successful conventional culture system for the isolation of CMV. The source of the fibroblasts can be either human embryonic tissues or newborn foreskin. The latter, however, lose their sensitivity after the 10th to 15th passage.

CPE may develop within a few days to many weeks, depending on the amount of virus in the specimen. Characteristic CPE consists of foci of enlarged, refractile cells that slowly enlarge over weeks and often do not involve the entire monolayer (Fig. 2C). Thus, it is important that the monolayers be maintained in good condition for at least 3 weeks. On the other hand, when a high titer of CMV is inoculated, as contained in urine samples from congenitally infected babies, one may see generalized rounding at 24 h that can be confused with HSV. For subculture, early passage of intact infected cells is essential. Monolayers should be trypsinized and then dispensed onto fresh uninfected cells. Identification of isolates can be accomplished with immunofluorescence. Since CPE is slow to advance, passage of trypsinized monolayers into centrifugation cultures followed by staining at 24 to 48 h can provide a more rapid confirmation.

Centrifugation cultures have had a major impact on the rapid diagnosis of CMV infections. However, for optimal CMV recovery from samples other than urine, conventional cultures should be performed in parallel (Rabella and Drew, 1990).

Mink lung cells, though not permissive for CPE in conventional cultures, have proved very useful for CMV centrifugation cultures, especially since ML cells are less susceptible to toxicity from blood samples (Gleaves et al., 1992).

Adenovirus

Throat swabs, nasopharyngeal swabs, eye swabs, and stool samples are good sources of virus, with the choice depending on the clinical syndrome.

In general, human adenoviruses produce CPE in continuous human cell lines, such as A549, and in HEK and in HDF cultures. Each of these cell systems has its disadvantages: the continuous cell lines may be difficult to maintain; HEK often are not readily available and are expensive; and the HDFs are less sensitive and the changes produced are not characteristic (Mahafzah and Landry, 1989). Nonhuman cells, such as RhMK infected with simian virus 40, are of variable sensitivity, and virus growth is slower.

Characteristic CPE consists of grape-like clusters of rounded cells (Fig. 2E), which appear in 2 to 7 days with types 1, 2, 3, 5, 6, and 7. Other adenovirus types may require 3 to 4 weeks or blind passage. Adenovirus is cell associated, similar to VZV and CMV; however, adenovirus is nonenveloped and stable to freezing and thawing. Therefore, two to three cycles of freezing at 70°C and thawing disrupts the cells and releases infectious virus. Enteric adenovirus types 40 and 41, associated with gastroenteritis, do not grow readily in A549 or HDFs, but can be isolated in H292 cells (Hierholzer et al., 1993).

Identification of isolates as adenoviruses can be done by IF using antihexon antibody. Neutralization tests with type-specific antiserum or molecular analysis will identify virus types.

Centrifugation cultures can provide a more rapid diagnosis, but staining at 2 days and again at 4 to 5 days may be needed for optimum sensitivity (Espy et al., 1987; Mahafzah and Landry, 1989; Van Doornum and DeJong, 1998).

Enterovirus

Enteroviruses were originally classified by their growth in different types of cell culture and suckling mice. With molecular sequencing, the identification of new strains, and development of new cell lines, enteroviruses have been reclassified into 4 groups (A to D) and echovirus types 22 and 23 have been moved to a new genus, *Parechovirus*.

Enteroviruses can be recovered from feces, throat swabs, cerebrospinal fluid, blood, vesicle fluid, conjunctival swabs, and urine. In general, enteroviruses grow best in epithelial cells of primate origin. Poliovirus and coxsackie B virus grow well in primary MK, HEp-2, and BGMK cells, and echovirus grows well in primary MK and RD (a rhabdomyosarcoma cell line) but not in HEp-2 cells (Table 6). The universal host for coxsackie group A is the newborn mouse; however, some strains grow in HDF, HEK, MK, or RD. Since inoculation of multiple cell types optimizes enterovirus detection (Dragan and Menegus, 1986; Kok et al., 1998), the use of mixed-cell cultures may result in greater yield while conserving time and resources.

Characteristically, infected monolayer cells round up, become refractile, shrink, degenerate, and then detach from the surface of the culture vessel (Fig. 1, panel 2). Virus in the supernatant fluid can be subpassaged. Preliminary

TABLE 6 Cell culture susceptibility for enteroviruses

Virus	Sensitivity in cell culture[a]:				
	RhMK	HDF	HEp-2	RD	BGMK
Poliovirus	+++	+++	+++	+++	+++
Coxsackievirus type A	+/–	++	–	+++	+/–
Coxsackievirus type B	+++	+	+++	+/–	++++
Echovirus	+++	+++	+/–	+++	++

[a]RD, human rhabdomyosarcoma cell line. Degree of sensitivity: ++++, maximally sensitive; +++, highly sensitive; ++, sensitive; +, less sensitive; +/–, variable; –, not sensitive.

identification can be determined by characteristic CPE and cell susceptibility (Fig. 3) (Johnston and Siegel, 1990a; Hsiung et al., 1994). Shell vial centrifugation cultures using multiple cell types (van Doornum and DeJong, 1998; She et al., 2006) or mixed cells (Buck et al., 2002) has shortened the time to detection. Fluorescein-labeled monoclonal antibodies available for identification of enteroviruses have been plagued with nonspecific staining (Rigonan et al., 1998). Newer monoclonal antibodies are reported to be more sensitive and specific (M. Vu, E. Yeh, and D. Schnurr, 23rd Annu. Clin. Virol. Symp., poster S-46, 2007).

Final identification and serotyping by microneutralization tests in cell culture using antiserum pools is expensive and time-consuming and has largely been replaced by PCR and sequencing (Oberste et al., 1999; Muir et al., 1998; Oberste et al., 2005).

Rhinovirus

Rhinoviruses are classified as picornaviruses along with the enteroviruses but can be separated from the latter by their sensitivity to low pH. Sources of virus include nasal swabs or washes and throat swabs.

Many rhinovirus types were originally isolated in organ cultures of human embryonic trachea. Rhinoviruses can be isolated in cells of human origin (usually HDFs), certain strains of HeLa cells, and human fetal tonsil cells; however, the varying sensitivity of different lots of cells can be a problem. WI-38 and HeLa-I cells have been identified as the most sensitive cells (Arruda et al., 1996), and cultivation at 33°C in a roller drum apparatus is optimal. Nevertheless, only a minority of rhinovirus infections are identified by cell culture. With wider use of molecular methods, the true prevalence of rhinovirus infections has been shown to be much greater than that shown by isolation in cell culture (Miller et al., 2007).

CPE may occur from the first to the third week of incubation. The CPE is similar to the enteroviruses, starts as foci of rounded cells, and spreads gradually (Fig. 1, panel 8). However, CPE may not progress and may even disappear; if it is not progressing, subpassage of supernatant fluids from infected cells should be performed. Isolates are identified by characteristic CPE and inactivation at pH 3. Typing by neutralization tests is reserved for research laboratories.

Influenza Virus

Nasopharyngeal aspirates and swabs, nasal washings, and throat swabs are good sources for virus and should be collected early in illness, preferably in the first 24 to 48 h. Primary MK is the most widely used cell culture for isolation of influenza, although the host range may be increased by the addition of trypsin to the medium (Frank et al., 1979). Madin-Darby canine kidney (MDCK), MRC-5, and ML (Schultz-Cherry et al., 1998) have all been used successfully, especially in centrifugation cultures (Reina et al., 1997). Influenza virus is also reliably isolated in eggs (Smith and Reichrath, 1974).

Serum components may inhibit influenza virus from replicating. Therefore, serum should be removed from cell cultures by rinsing with Hanks' balanced salt solution before inoculation, and cultures should be maintained in serum-free medium after inoculation. Incubation at 33°C in a roller drum is optimal for isolation. The presence of virus is generally detected by hemadsorption of guinea pig RBC onto infected monolayers (Fig. 4B). CPE is seen with influenza (Fig. 1C), but it usually occurs later than the detection of virus by hemadsorption. Subcultures can be performed by passaging the supernatant fluids. Isolates can be identified as influenza A or B virus by IF using monoclonal antibodies. Subtype and strain identification are traditionally determined by hemagglutination inhibition, although subtyping by monoclonal antibody staining is feasible (Tkacova et al., 1997) and reverse transcriptase PCR and other molecular methods are becoming more widely used.

Parainfluenza Virus

Nasopharyngeal aspirates and swabs, nasal washings, and throat swabs are good sources for virus. Primary MK is the most sensitive system for isolation. HEK, HDFs, and HEp-2 are less sensitive. Some success has recently been reported with H292 cells (Hierholzer et al., 1993).

Cell cultures should be washed with Hanks' balanced salt solution before inoculation and refed with medium without serum. Incubation at 33 to 36°C in a roller drum is optimal. The presence of virus is detected by hemadsorption (Fig. 4B), which occurs before CPE. Parainfluenza virus type 2 may produce syncytia, especially in HEp-2 cells. On subculture, parainfluenza virus type 3 also may induce syncytium formation. In those instances when high levels of virus are present, hemadsorption may be detected in the infected cultures within a few days; with specimens containing less virus, 10 days or more of incubation may be necessary. Identification is by IF or by hemadsorption inhibition.

RSV

RSV is found in respiratory secretions from the nose and oropharynx. Sample collection is important, and RSV is more reliably detected from nasopharyngeal aspirates than from swabs in children (Ahluwalia et al., 1987). RSV grows best in continuous cell lines, such as HEp-2, in which it produces characteristic syncytia (Fig. 1, panel 6). However, syncytium formation is variable, and viral replication may be missed. If HEp-2 cells are confluent and 5 to 7 days old when inoculated, syncytia may not form. Rather, nonspecific rounding may occur. Syncytium formation is also dependent upon the presence of adequate levels of glutamine and calcium in the medium (Marquez and Hsiung, 1967; Shahrabadi and Lee, 1988). Primary MK cells and HDFs support RSV growth, but the cytopathology is not as characteristic (Arens et al., 1986). HEp-2 cells have become so difficult to work with that many laboratories rely on other cell cultures, with suboptimal results. Identification is by IF, and centrifugation culture can improve detection.

ADVANTAGES AND LIMITATIONS OF CELL CULTURE

The advantages of cell culture for virus diagnosis include broad-spectrum, biologic amplification of the input virus, ability to detect infectious virus, greater sensitivity than antigen detection methods, and the recovery of unknown or unexpected infectious virus(es) present in the specimen (Mackenzie, 1999). It is limited by the inherent time delay required for virus growth, by the difficulty in maintaining cell cultures, by the sometimes variable quality of cultures, and by the decreased sensitivity of cell lines at higher passage levels. Some common viruses as yet do not produce identifiable effects in readily available cell cultures. Examples include hepatitis viruses, noroviruses, and some group A coxsackieviruses.

Contamination with adventitious agents occurs, including bacteria, fungi, viruses, and mycoplasma, which can inhibit

the growth of viruses in clinical specimens or destroy the cell culture (Hsiung, 1968; Smith, 1970; Stanbridge, 1971; Chu et al., 1973; Whiteman, 2006). Endogenous viruses that are latent in the tissue culture or calf serum can be reactivated during cultivation, cause CPE or hemadsorption, and thus, be confused with virus isolated from the patient's specimen (Fong and Landry, 1992). Viruses potentially contaminating primary monkey kidney cell cultures, such as herpes B virus, can pose a serious health risk as well.

The presence of inhibitory substances and/or antibodies in calf serum used in the cell culture media can reduce the isolation of certain viruses, especially of the orthomyxovirus and paramyxovirus groups (Krizanova and Rathova, 1969). Ideally, maintenance media for inoculated cultures should be serum-free; however, serum is required for long-term maintenance of cells. Using fetal or agammaglobulin calf serum reduces this problem but adds to expense. To date, no completely satisfactory, chemically defined medium is available (Merten, 2002).

To get the best results from primary isolation in cell culture, healthy cell cultures susceptible to a spectrum of viruses are essential. Traditionally, this has required the inoculation of at least three separate cell cultures, such as a primary monkey kidney cell culture, an HDF strain, and a human heteroploid cell line (e.g., A549), with observation for CPE for 1 to 3 or 4 weeks. However, if patient management is to be affected, results must be available quickly.

Reducing the time to result has become increasingly important for reducing unnecessary tests and antibiotics, initiating antiviral therapy, implementing infection control measures, and shortening hospital stays. The need to reduce costs and do more with fewer personnel has created additional pressures. Innovations in viral culture methods that have reduced turnaround time and labor and allowed both positive and negative cultures to be reported in 48 h have included rapid shell vial centrifugation cultures, use of pooled antibodies for detection of multiple viruses, mixtures of two cell systems in one culture vessel, and genetic engineering to enhance cell culture susceptibility to particular viruses. The greater use of continuous cell lines and more stringent quality control has reduced the incidence of adventitious agents in the cultures. Furthermore, cryopreserved cell cultures can now be purchased and stored at –70°C and thawed as needed for inoculation of clinical samples, thus making culture more economical and user-friendly. The ELVIS HSV culture system can be used by laboratories that do not have experience in detection of CPE but want to provide HSV detection.

Cell culture continues to facilitate the discovery and rapid molecular characterization of new viruses, such as human metapneumovirus (van den Hoogen et al., 2001), NL63 coronavirus (van der Hoek et al., 2004; Fouchier et al., 2004), and SARS coronavirus (Ksiazek et al., 2003).

Although molecular methods are increasingly used, especially in reference laboratories and larger tertiary care centers, many hospitals have limited in-house molecular diagnostic capability. Since cultures can be inoculated every day, the time to result can be faster than molecular methods that are not performed as frequently or are sent to a distant reference laboratory. Detection of infectious virus in culture may have a better predictive value for clinical significance than highly sensitive molecular methods. Virus isolation is generally more sensitive than rapid antigen detection assays, detects a broader spectrum of viruses, and is less costly than molecular assays. Although conventional virus isolation with observation for CPE has more limited application in the diagnostic laboratory than a decade ago, recent cell culture innovations have increased viral diagnostic capabilities, shortened turnaround times to 1 to 2 days in most cases, and significantly reduced the technical expertise, labor, and quality control required. Thus, cell culture methods continue to play an important role in virology testing, especially when performed onsite for hospitalized patients and immunocompromised hosts.

Ultimately, the combination of culture and nonculture methods used will depend on laboratory size, expertise, resources, and clinical usefulness in each setting. For laboratories that perform the simple rapid RSV and influenza virus antigen tests, mixed-cell cultures provide a broader-spectrum diagnosis with significantly enhanced sensitivity and specificity, and importantly, results in 1 to 2 days. For those laboratories that rely on direct IF for rapid diagnosis of herpes and respiratory viruses, conventional cell culture methods remain essential in establishing, validating, and monitoring the performance of these tests. Furthermore, either rapid or conventional culture methods can be the backup for testing samples that are inadequate for IF or when greater sensitivity is needed. In addition, conventional culture should be used for lower respiratory tract and tissue biopsy samples to detect additional or unsuspected viruses. While molecular methods are essential for optimal detection of viruses in spinal fluid and to monitor viral load in blood, virus isolation continues to play an important role in viral diagnosis and patient management, especially when performed locally.

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The Cytopathology of Virus Infection

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4

Virus-infected cells that exfoliate or are scraped from the skin or mucous membranes may contain readily identifiable morphologic changes that permit rapid diagnosis. In many instances, the cytologic alterations may be so distinctive as to be pathognomonic for infection with a specific agent; in others, the changes may point to a virus group or merely raise a suspicion of infection to be confirmed by other means, such as immunohistology and/or in situ hybridization. The purpose of this chapter is to describe the methods used to obtain and prepare cells for cytologic examination and to illustrate characteristic changes encountered in common virus infections. Most of the methods described are used routinely by diagnostic cytology laboratories and are best applied by the cytotechnologist or pathologist who analyzes cytologic material on a daily basis.

PREPARATION AND STAINING

The proper collection, fixation, preparation, and staining of specimens for cytology is essential. Rapid fixation in 95% alcohol or with cytology spray fixative before the smear dries is imperative for accurate interpretation. The purpose of fixation is to maintain the existing form and structure of the cellular elements and to achieve consistent staining characteristics and identifiable structures. Improper specimen preparation will decrease the diagnostic accuracy and may lead to false-positive or false-negative results. Air drying causes nuclear swelling and distortion, cytoplasmic vacuolization, and atypical staining. These changes can mimic nuclear and/or cytoplasmic alterations seen with some virus infections or can distort the characteristic details to such an extent that viral cytopathology cannot be identified. For example, the ground-glass nuclear appearance seen in early herpes simplex virus (HSV) infection can be confused with the smudgy nuclear detail seen in air-dried specimens. Aqueous fixatives (e.g., formalin) result in poor staining and irregular condensation of chromatin, which can be mistaken for a nuclear inclusion.

The preparatory methods utilized for the microscopic examination of cytologic specimens can be divided into five categories: direct smears, preparation by cytocentrifugation, membrane filter preparation, monolayer preparation, and cell block preparation.

Direct Smears

Direct scraping of vesicular or bullous lesions of the skin and mucous membranes is the simplest method of cell collection for the identification of viral changes. Proper sampling is important because the crusted or eczematous areas often fail to show the diagnostic cellular features. If a specific lesion, such as an ulcer or unroofed vesicle, is present, the base and edges of the lesion should be thoroughly scraped with a spatula, tongue blade, or endoscopic brush to ensure proper sampling. The scrapings from the base and edges of the suspected lesion should be smeared evenly onto a clean glass slide and immediately fixed with 95% alcohol or cytology spray fixative. Once fixed, the smears are almost indefinitely stable at room temperature and can be stored or transported to a cytology laboratory for further processing. The slides are then stained with a modified Papanicolaou staining technique.

Liquid-Based Preparations

Since its introduction in 1996, liquid-based cytology has come to dominate the cytologic analysis of specimens. While direct smears are still used and are useful, liquid-based preparations make up nearly 90% of specimens. In nongynecologic cytology, specimens are routinely sent in liquid preservative, which is alcohol based, with or without a slide or slides from direct smears. After collection, the specimen is centrifuged to concentrate it, and then the concentrate is resuspended in the preservative solution and processed on an automated device.

When received for processing, specimens are placed on a processor that disperses the cells and then collects them on a filter. The filter is then pressed against a slide, transferring the concentrated and randomized cells in a monolayer for microscopic examination. An alternative liquid-based method also uses a centrifuged specimen but follows with sedimentation of the cells by gravity rather than cell transfer with a membrane.

Modified Papanicolaou Stain

The modified Papanicolaou stain technique is used to detect cytologic changes due to viral infection. The procedure for staining is as follows:

1. Ten dips in 95% ethyl alcohol (EtOH)
2. Ten dips in 70% EtOH
3. Ten dips in 50% EtOH
4. Ten dips in distilled H_2O
5. Two minutes in hematoxylin (Gill hematoxylin, consisting of 2,190 ml of distilled H_2O, 750 ml of ethylene glycol, 6 g of hematoxylin, 0.6 g of sodium

iodinate, 528 g of aluminum sulfate, and 60 ml of glacial acetic acid)
6. One minute under running tap water
7. One minute in Scott's tap water substitute (consisting of 10 g of anhydrous magnesium sulfate, 2 g of sodium bicarbonate, and 1 l of distilled water)
8. Ten dips in tap water
9. Ten dips in 50% EtOH
10. Ten dips in 95% EtOH
11. One and one-half minutes in OG-6'
12. Ten dips in 95% EtOH
13. Ten dips in 95% EtOH
14. Three minutes in EA-65'
15. Ten dips in 95% EtOH
16. Ten dips in 95% EtOH
17. Ten dips in 95% EtOH
18. Ten dips in 100% EtOH
19. Ten dips in 100% EtOH
20. Ten dips in 100% EtOH–Hemo-De[2] (equal amounts)
21. Ten dips in Hemo-De, in three consecutive dishes
22. Coverslip slide with Permount media

Cytocentrifugation and Filtration

New cytocentrifugation and filtration techniques have been developed which concentrate small numbers of cells suspended in fluids and are the preferred method for preparation of samples from urine, cerebrospinal fluid, bronchial wash and bronchial alveolar lavage (BAL) fluid, and body cavity fluids. To determine which of the two techniques should be used, one needs to consider the expected number of cells in the fluid. Fluids containing many cells should be centrifuged. The specimen is placed in a centrifuge tube and spun at 1,500 rpm for 10 min. The supernatant fluid is decanted, leaving a volume of 2 ml in the tube, and the sediment is resuspended. The specimen is then prepared using the standard cytocentrifugation technique (Barrett, 1976). If little or no sediment is present, then the filtration technique (Gill, 1976) is the method of choice. The use of membrane filters should be limited to cases where the number of cells is low and where additional cell sampling presents a problem.

Cytospin preparations offer several advantages. The cells are evenly dispersed on the slide (monolayer), little or no background artifact is present, and the cell preparation can be utilized easily for other diagnostic procedures, such as special stains, immunofluorescence, immunoperoxidase, and in situ hybridization using virus-specific RNA or DNA embedded probes. Cell block preparations require a histopathology laboratory and are used when there is an abundance of cellular material that can be embedded in paraffin and sectioned for histologic examination.

VIRUS CYTOPATHOLOGY

The eye and respiratory, genital, and urinary tracts are locations that readily yield cytologic material for rapid viral diagnosis. Characteristic cytologic changes depend on the cytopathic effect of a virus in infected cells, which need not include all cells of the involved organ. In practice, however, it is most useful to consider cytologic alterations in the context of the organ system affected and the clinical presentation. Therefore, the following discussion and illustrations are organized according to organ system.

Viral Infections of the Respiratory Tract

Smears of cells obtained by nasal and throat swabs, tracheal aspirates, sputum, bronchial washings and brushings, and BAL fluid may exhibit cytologic alterations that are diagnostic of virus infection (Table 1).

In adults, the most frequently encountered virus that is readily detectable by cytology is cytomegalovirus (CMV) (Warner et al., 1964). Particularly in patients who are receiving immunosuppressive therapy for transplantation or cancer chemotherapy and in AIDS, the rapid cytologic identification of characteristic inclusions may be a great asset in patient management, particularly because CMV isolation in cell culture takes many days and often weeks. Because CMV infection involves the lungs, a deep specimen containing pulmonary macrophages is needed. Patients with CMV pneumonia rarely produce abundant sputum, thus requiring bronchial washings and brushings or BAL fluid to obtain an adequate specimen. The characteristic cytologic changes are seen in pulmonary macrophages or in cells lining the alveoli. They most often exhibit a single nucleus, but occasionally there are two or more nuclei that are four to six times their normal size (Fig. 1). Early in the infection these nuclei contain amphoteric or basophilic inclusions that are granular, and the inclusions become condensed and surrounded

TABLE 1 Cytopathology of respiratory viral infections

Virus	Clinical presentation	Cytologic findings
Adenovirus	Upper respiratory infection, pneumonia	Small multiple eosinophilic IN inclusions (early); large, single, dense basophilic IN inclusion (late)
CMV	Pneumonia	Cytomegaly; large, single or occasionally binucleate, amphophilic IN inclusions; small periodic acid-Schiff reaction-positive IC inclusion
HSV	Tracheobronchitis, pneumonia	Large ground-glass nucleus (early); eosinophilic IN inclusions (late); multinuclearity with nuclear molding
Parainfluenza virus	Bronchitis, pneumonia	Cytomegaly, single nucleus, small eosinophilic IC inclusions
RSV	Tracheobronchitis, pneumonia	Large multinucleated cells; IC basophilic inclusions with prominent halos
Measles virus	Prodromal	Mulberry-like clusters of lymphocytic nuclei in nasal secretions
	Pneumonia	Multinucleated giant cells with IN and IC eosinophilic inclusions
Nonspecific (many viruses)	Bronchitis, pneumonia	Ciliocytophthoria

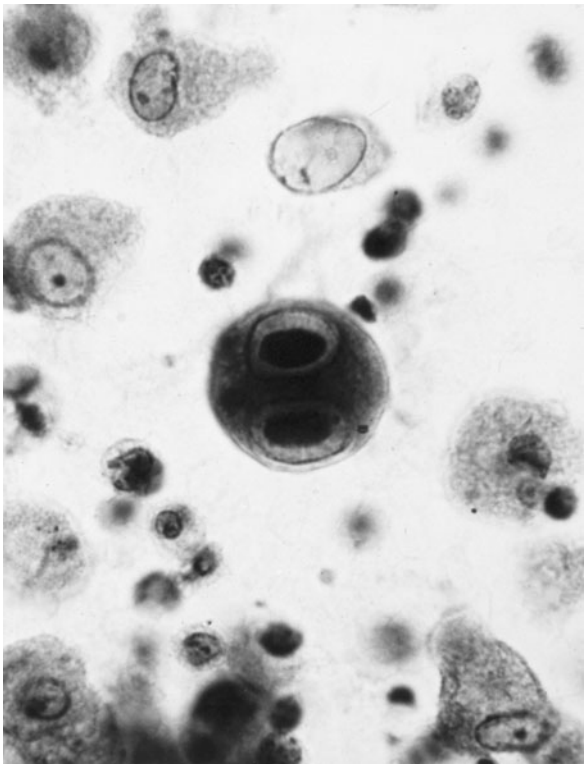

FIGURE 1 CMV in bronchial brushing; large basophilic IN inclusions in large binucleated cell with small IC inclusions; Pap stain. Magnification, ×800.

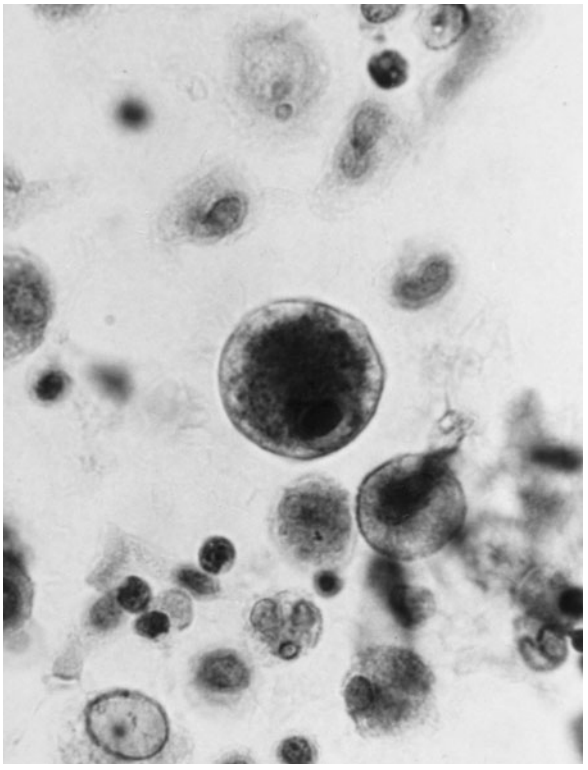

FIGURE 2 CMV in urine; multiple IC inclusions with small degenerating IN inclusion in late stage of infection; Pap stain. Magnification, ×800.

by a halo in the later stages of infection. Smaller, more eosinophilic oval intracytoplasmic (IC) inclusions that are periodic acid-Schiff reaction positive are often, but not invariably, present. At later stages of the infection, IC inclusions may predominate with an empty or collapsed nucleus (Fig. 2).

Characteristic inclusion-bearing cells may be observed in sputum and bronchial washings in HSV tracheobronchitis, which is encountered often in immunosuppressed and burn patients (Vernon, 1982). Inclusion-bearing cells tend to be multinucleated and contain either eosinophilic intranuclear (IN) inclusions that are centrally located and surrounded by a halo or, at an earlier stage of infection, ground-glass inclusions that stain poorly (Fig. 3). The chromatin often appears as a basophilic ring condensed at the periphery of the nuclear membrane. IC inclusions are not present. When there are multiple nuclei, they are frequently molded or indented by each other (Fig. 4). Because HSV tends to produce cellular necrosis, the background of these smears usually contains an abundance of cellular debris.

Adenovirus infections of the upper respiratory tract may be identified by smears of secretions from the nasopharynx. Adenovirus pneumonia may be diagnosed by finding typical IN inclusions in bronchial or epithelial cells obtained by bronchoscopy. At an early stage (Fig. 5), the nucleus contains multiple small, rounded eosinophilic inclusions, each surrounded by a halo. At a later stage, a single larger, dense, basophilic IN inclusion is seen (Fig. 6).

Respiratory syncytial virus (RSV) and parainfluenza viruses frequently cause bronchitis and pneumonia in infants and young children They can be rapidly diagnosed by identifying characteristic cytologic changes in respiratory

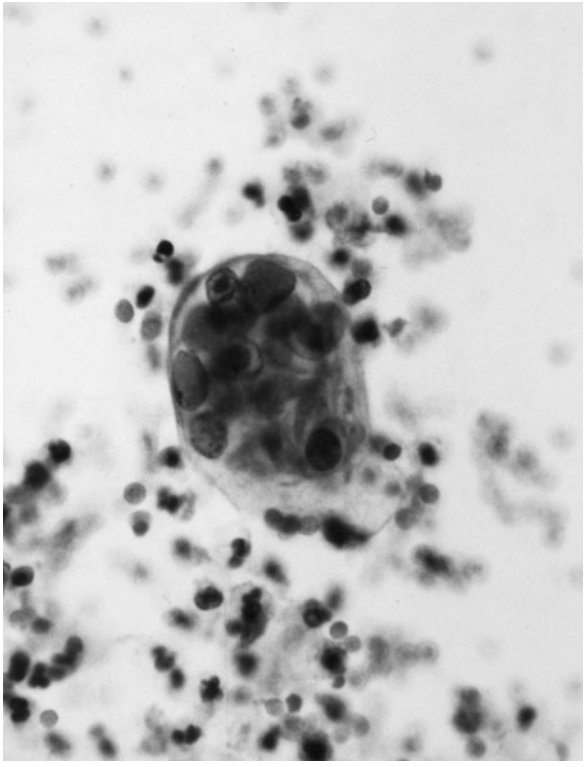

FIGURE 3 HSV in sputum; multinuclear cell with IN inclusions in different stages of development. Magnification, ×600.

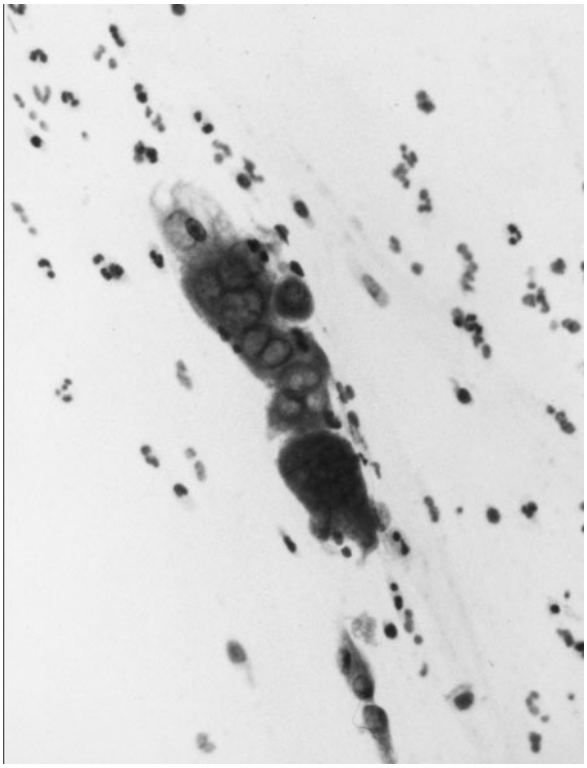

FIGURE 4 HSV in bronchial brushing; multinucleated cell with IN inclusions having a ground-glass appearance and showing nuclear molding; Pap stain. Magnification, ×800.

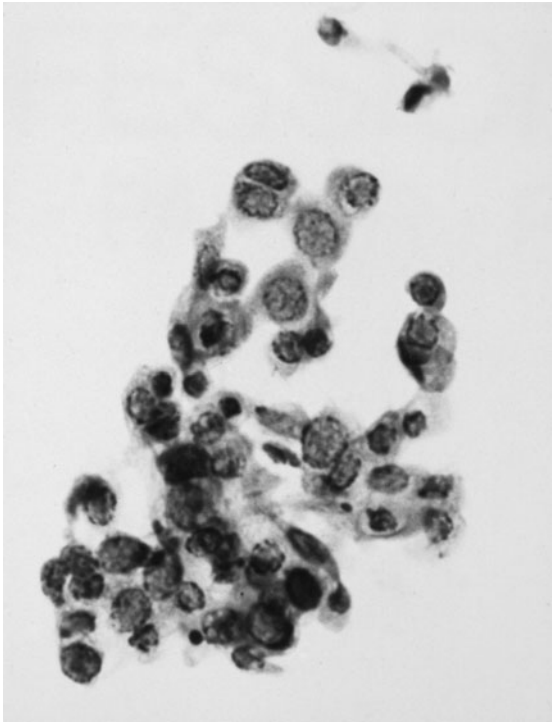

FIGURE 5 Adenovirus in conjunctival scraping; granular IN inclusions with condensed chromatin at the nuclear membrane, characteristic of the early stage of infection; Pap stain. Magnification, ×600.

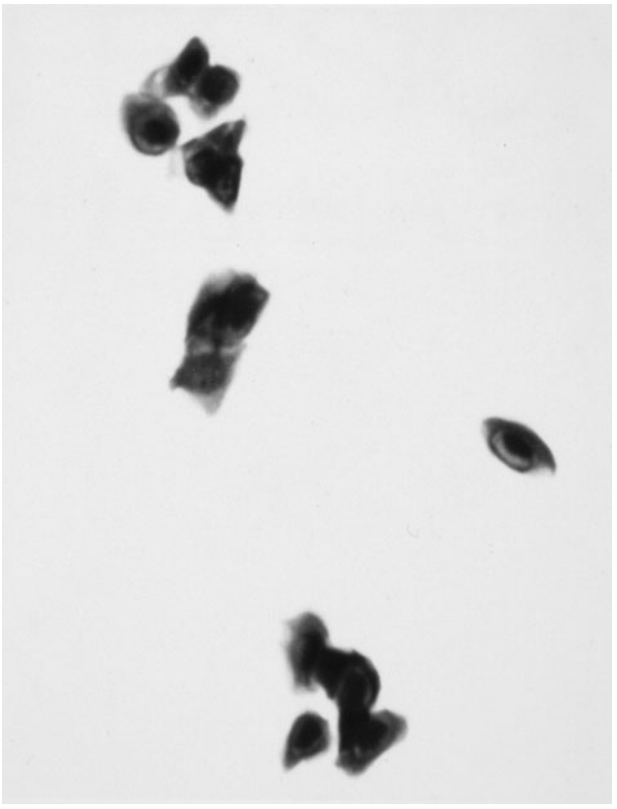

FIGURE 6 Adenovirus in conjunctival scraping; cells contain a dense, sometimes teardrop-shaped IN inclusion surrounded by a clear halo in the late stage of infection; Pap smear. Magnification, ×800.

epithelial cells obtained by nasopharyngeal swabs and tracheal aspirates (Naib et al., 1968). Parainfluenza can be differentiated from RSV by finding large cells containing a single nucleus and multiple small eosinophilic inclusions. In RSV infection, the epithelial cells are large and multinucleate and the cytoplasm contains multiple basophilic inclusions with prominent halos.

Measles can be detected during the prodrome by finding mulberry-like clusters of lymphocytes having up to 50 nuclei in smears of nasal secretions (Tomkins and Macaulay, 1955). Measles giant cells (Fig. 7) are multinucleate respiratory epithelial cells with IN and IC inclusions. These cells may appear in the sputum of patients with measles pneumonia.

A nonspecific change referred to as ciliocytophthoria is found in various inflammatory diseases of the respiratory tract and, in particular, virus infections (Pierce and Hirsch, 1958). The ciliated bronchial epithelial cells undergo a degenerative process in which a pinching off occurs between the cytoplasm and nucleus, resulting in detached tufts of cilia, and a degenerating nucleus and cytoplasm (Fig. 8). The degenerated cytoplasm may contain small, round eosinophilic inclusion bodies (Takahashi, 1981). Ciliocytophthoria occurs most frequently with influenza virus, parainfluenza virus, and adenovirus infection but may also occur in bronchiectasis and other nonviral inflammatory conditions. It is seen more frequently in sputum specimens than in those obtained by bronchoscopy.

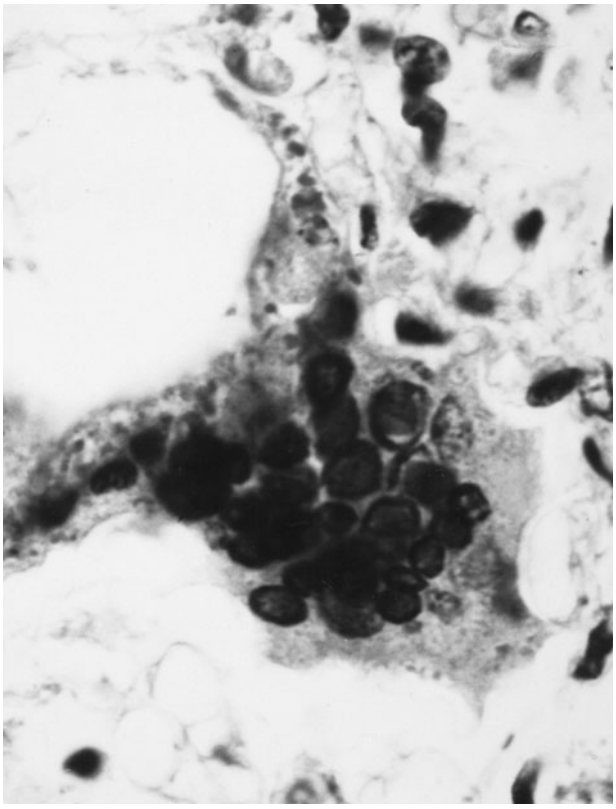

FIGURE 7 Measles pneumonia; paraffin-embedded lung tissue showing a multinucleated giant cell with multiple IN and IC inclusions; hematoxylin and eosin stain. Magnification, ×800.

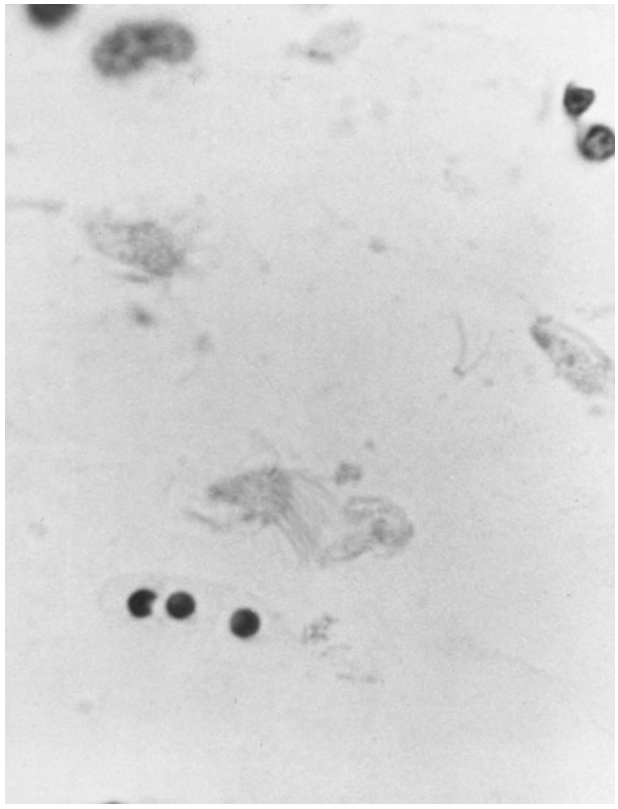

FIGURE 8 Ciliocytophthoria in sputum; a tuft of detached cilia and nuclear debris; Pap stain. Magnification, ×600.

Virus Infections of the Urinary Tract

Although many viruses that cause systemic infections have been isolated from the urine, those most readily diagnosed by urine cytology are CMV, HSV, and a member of the papovavirus group, designated the BK virus (BKV) (Table 2).

In each of these infections, epithelial cells of the urinary tract from the renal tubules to the bladder and urethra detach and enter the urine. These cells often contain characteristic inclusions. Because of the relatively small number of cells in a large fluid volume, filtration or cytocentrifugation is necessary to obtain a suitable preparation (Schumann et al., 1977). Each of these infections occurs most often, but not exclusively, in immunosuppressed patients (commonly renal transplant recipients) and may coexist as mixed infections. CMV-infected urothelial cells were first described in the urine of newborn infants with cytomegalic inclusion disease (Fetterman, 1952). Cytologic examination of the urinary sediment is indicated with any infant suspected of having neonatal CMV infection. Positive cytology is seen in approximately 50% of neonates that will subsequently have CMV-positive cultures (Hanshaw et al., 1968). CMV is the most frequently encountered viral infection in renal transplant recipients. In one study, it was found in 31% of 2,354 cytologically examined routine urine samples obtained from 91 patients (Traystman et al., 1980). Cellular changes include cytomegaly of the urothelial cells with typical large IN inclusions surrounded by a clear halo and smaller eosinophilic IC inclusions. This cytopathology most often involves single cells (Fig. 2). Although the classical large, dense, IN inclusion is the easiest to identify, occasional cells may be binucleated and some cells may have large dense eosinophilic IC inclusions with little or no evidence of an IN inclusion.

BKV was first isolated from the urine of a 39-year-old man who developed ureteral stenosis 4 months after renal transplantation (Gardner et al., 1971). The urine sediment contained abnormal transitional cells with dense IN inclusions composed of crystalline arrays of papova virions as revealed by electron microscopy. BKV was later isolated from many asymptomatic transplant recipients (Coleman, 1975) and two other patients with ureteral stenosis (Coleman et al., 1978). At an early stage, the most recognizable cytologic change due to BKV infection is the enlarged nucleus in an epithelial cell that contains a mucoid inclusion filling the nucleus (Fig. 9). A more homogeneous, densely basophilic inclusion is detached at a later stage (Fig. 10). This is sometimes referred to as a "decoy cell." Frequently, the nucleus appears to be bulging from the cytoplasm or thrusting from it, giving the infected cell a comet-like appearance (Fig. 11). Although most involved cells have single nuclei, occasional binucleated forms are seen with both types of inclusions present. At a later stage, the inclusion shrinks from the nuclear membrane, leaving an incomplete thin halo. The IN inclusions of BKV can be distinguished from those of CMV by the complete and consistent halo around the CMV inclusion and the lack of IC inclusions in BKV (compare Fig. 1 with Fig. 10). BKV infection of the kidney is frequently observed in biopsy

TABLE 2 Virus infections of the urinary tract

Virus	Clinical presentation	Cytologic findings
Adenovirus	Hemorrhagic cystitis	Dense basophilic IN inclusions in transitional cells
BKV (human polyomavirus)	In renal transplant patients with signs of rejection and in other asymptomatic immunosuppressed patients	Large full mucoid IN inclusions (early); dense full basophilic inclusions bulging from cytoplasm (late)
CMV	Asymptomatic or polysymptomatic in immunosuppressed patients	Large basophilic IN inclusions surrounded by halo; multiple eosinophilic IC inclusions
HSV	Generalized infection or local cystitis; may be contaminant from herpes genitalis	Ground-glass nuclei (early); eosinophilic IN inclusions (late); multinuclearity, may be part of tubular cast
Measles virus	Measles with exanthema	Multinucleated giant cells with IC inclusions

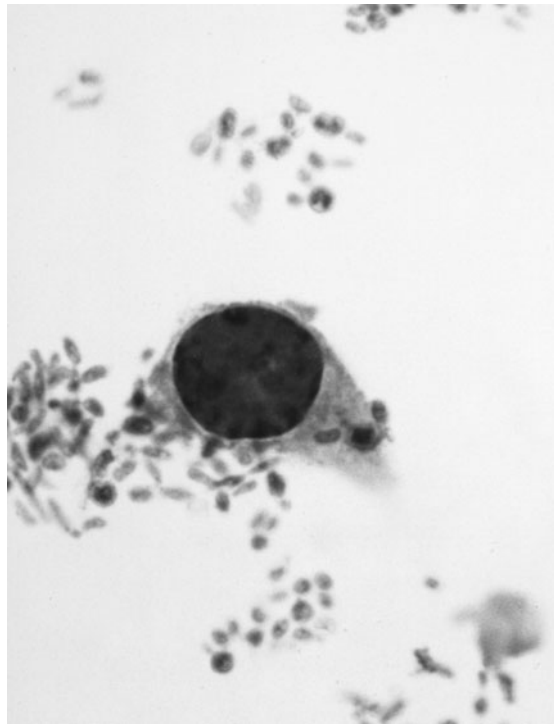

FIGURE 9 BKV polyomavirus in urine; uroepithelial cell with early IN inclusion. The enlarged nucleus is filled with a grayish-staining inclusion having a mucoid appearance. The background cells are yeast. Pap stain. Magnification,×800.

specimens of renal allografts. The presence of characteristic inclusion-bearing cells may necessitate urgent treatment or the transplant may be lost from BKV nephritis.

Cytologic changes of HSV-infected cells in the urinary sediment are similar to those described for the respiratory tract. They include multinuclear syncytial cells with enlarged ground-glass nuclei, seen at an early stage of infection, and typical eosinophilic IN inclusions surrounded by a halo at a later stage. Elongated clumps of infected epithelial cells, probably of tubular origin, may contain inclusions in varying stages of development (Fig. 12). Urinary sediment cells characteristic of HSV may occur in a generalized HSV infection involving the kidney or a localized cystitis; they also may result from herpes genitalis, particularly when there is vaginal infection (Masukawa et al., 1972). Other cytologic changes that may be observed in urinary sediment cells include IN inclusions of adenovirus associated with acute hemorrhagic cystitis in children (Numazaki et al., 1973) and inclusion-bearing cells in the urine of patients with measles (Bolande, 1961).

Virus Infections of the Genital Tract

Cytologic recognition of typical viral changes in cells of routine Pap smears is the most readily available and cost-effective method of detecting genital herpes infections (Table 3). Detection of HSV genital tract infection is important in abating the spread of sexually transmitted HSV as well as protecting the neonate from life-threatening infection transmitted to the infant during a vaginal birth. Cytologic recognition of HSV is of critical importance in directing the management of pregnancy near term. The

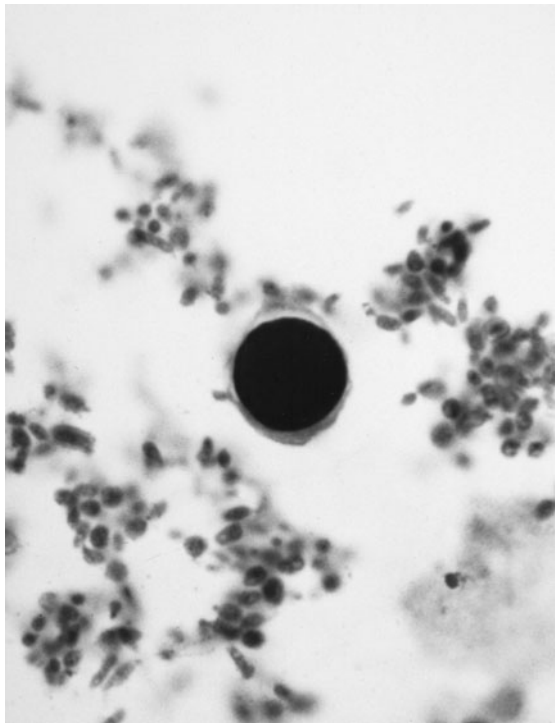

FIGURE 10 BKV in urine. A cell, probably from the renal tubules, contains a large, dense IN inclusion filling and expanding the nucleus. The background contains yeast. Pap stain. Magnification,×800.

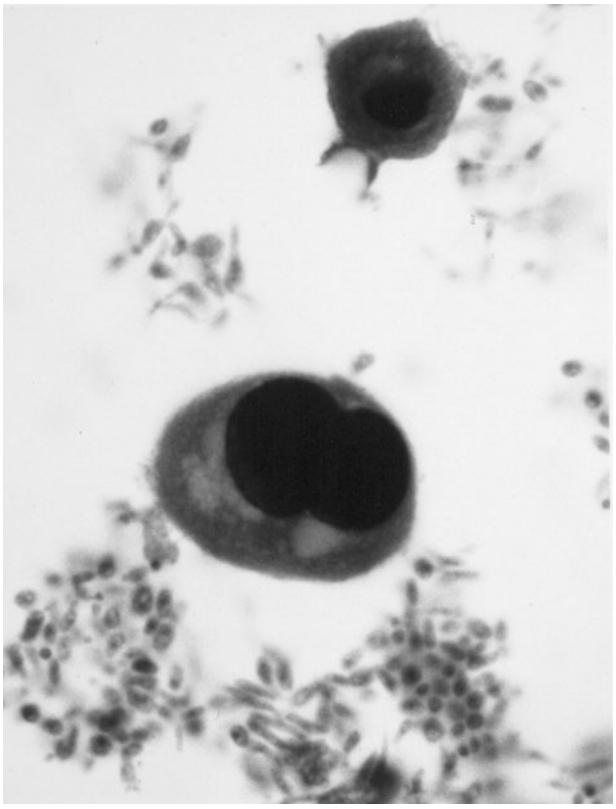

FIGURE 11 BKV in urine. In the late stage, there is cytoplasmic degeneration, leaving dense IN inclusions appearing to bud from the nucleus. Pap stain. Magnification, ×800.

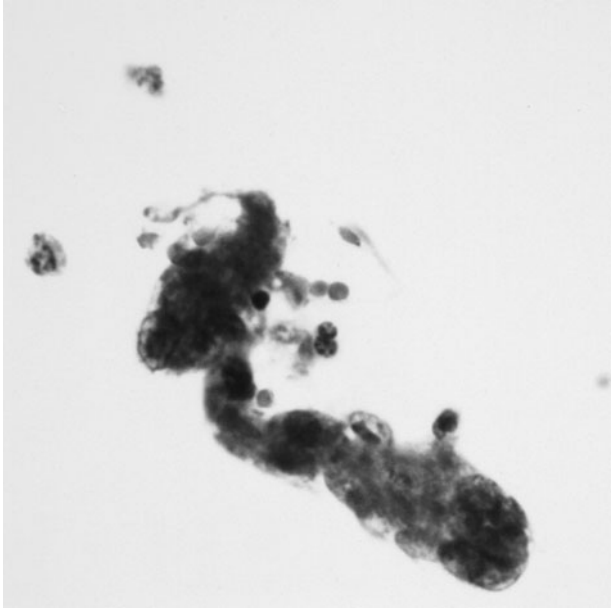

FIGURE 12 HSV in urine; a renal tubular cast containing characteristic HSV inclusions with nuclear molding; Pap stain. Magnification, ×800.

TABLE 3 Virus infections of the genital tract

Virus	Clinical presentation	Cytologic findings
HSV (types 1 and 2)	Herpes genitalis	Ground-glass nuclei (early); eosinophilic IN inclusions (late) with peripheral chromatin condensation; multinuclearity
Papillomavirus	Condyloma acuminatum; cervical dysplasia	Enlarged hyperchromatic nucleus; rare basophilic IN inclusions; perivascular cytoplasmic clearing and vacuolar degeneration (koilocytotic change)
Molluscum contagiosum virus	Vaginal, penile, or perineal papule with central umbilication	Large densely staining IC inclusions displacing nucleus; squamous cell often bean shaped

overall incidence of HSV in routine vaginal smears has been reported at approximately 3.0% (Naib, 1980), although this figure varies greatly depending on the patient population. The sensitivity of cytology for detecting HSV infection depends somewhat on the location of the herpetic lesions and the adequacy of the sample. In one study (Vontver et al., 1979), 41% (28/69) of cases with external lesions that were virus isolation positive had positive smears, but the rate was 23% with women that had only cervical lesions. Similar results were reported in a study of 76 patients with genital HSV comparing virus isolation, immunofluorescence, immunoperoxidase, and cytology as means of making a diagnosis (Moseley et al., 1981). The overall positive cytology rate was 37.6%, but it was 47.9% for cases with vaginal or cutaneous lesions. Significantly, there were a number of cases in which the cytology was positive, but virus isolation in cell culture from the same sample was negative. This discrepancy is repeatedly encountered in reported studies comparing virus isolation and cytopathology with various viruses at different sites and indicates that the greatest diagnostic yield with virus amenable to cytologic diagnosis is from the combination of cytology and virus isolation.

The identifiable cytologic changes in genital HSV infection are identical to those described with infections of the respiratory and urinary tracts. At an early stage, the enlarged nuclei have a bland ground-glass appearance with the chromatin displaced to the periphery, resulting in an apparent thickening of the nuclear membrane (Fig. 13). At a later stage, the nucleus contains an eosinophilic inclusion surrounded by a clear halo. Multinuclear cells are common with up to 10 nuclei, which often exhibit molding. Inclusions in multinucleated cells may all be at the same stage (Fig. 14) or may exhibit different stages of development (Fig. 15). HSV-1 and HSV-2 produce identical morphologic changes and cannot be differentiated on the basis of cytology. Although it has been reported that primary HSV

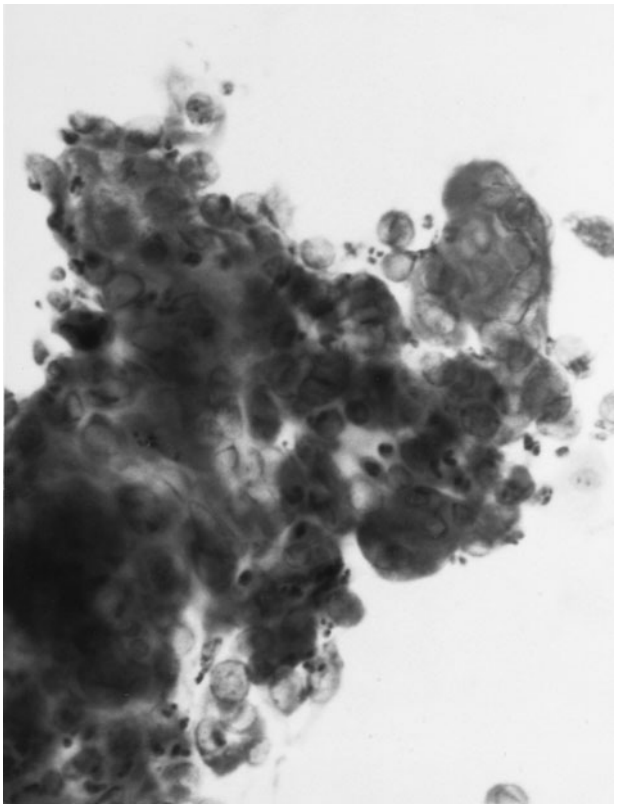

FIGURE 13 HSV in genital smear. At the early stage of the infection, there are ground-glass-appearing IN inclusions in a multinucleated mass of cells. Pap stain. Magnification, ×600.

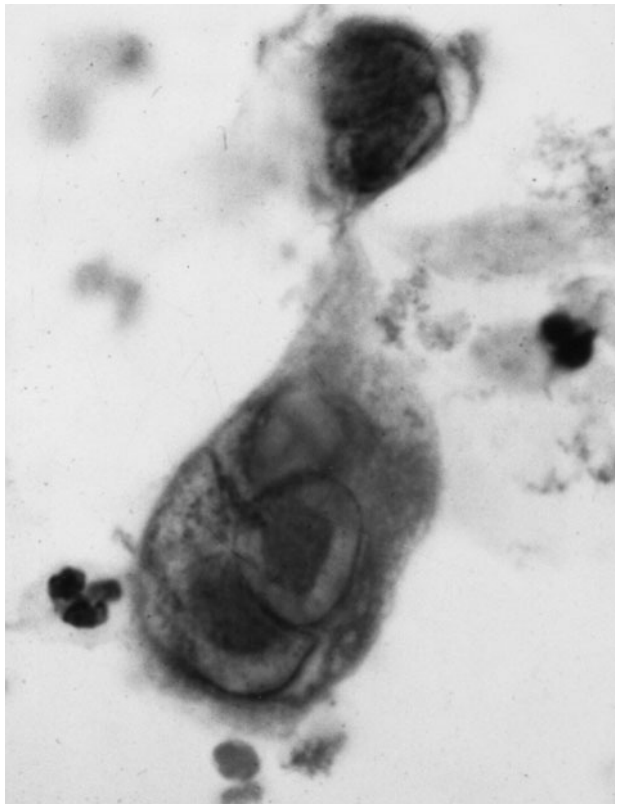

FIGURE 14 HSV in genital smear. A multinucleated cell contains angulated IN inclusions surrounded by halos. There is condensation of chromatin at the nuclear membrane and nuclear molding. Pap stain. Magnification, ×800.

infection can be differentiated from recurrent or secondary infection by a predominance of bland ground-glass nuclei in primary infection (Ng et al.,1970), this has not been confirmed in subsequent studies (Naib, 1980).

Infection of the cervical or vaginal mucosa and the skin of the perineum with a human papillomavirus (HPV) may result in proliferation of epithelial cells forming a vegetative papillary growth known as a condyloma acuminatum or venereal wart. Atypical cellular changes often accompany the proliferative process and may result in cellular alterations similar to those of malignant cells (Meisels et al., 1981). HPV has been associated with premalignant cervical dysplasia and carcinoma and identified by immunohistology and/or molecular probes in association with squamous cell carcinoma of the cervix and vulva (Zachow et al., 1982; Pilotti et al., 1984). At the present time, cytology is the only readily available practical way of identifying an HPV infection of the genital tract when a characteristic gross condylomatous lesion is not observed. While nucleic acid hybridization and amplification assays are also used to identify HPV infection, they are currently performed only after a cytologic abnormality is identified, as in an ASC-US (atypical squamous cells [ASC] of undetermined significance) Pap test. Although these molecular methods currently serve as an adjunct to the Pap test, molecular HPV testing has been studied as a screening tool in areas of Latin America and may eventually precede or replace routine Pap smears (Longatto-Filho, 2006). The changes attributable to HPV infection involve squamous cells, which appear swollen and have a perinuclear halo with poor cytoplasmic keratinization resulting in irregular staining. This produces a picture referred to as koilocytotic change (Fig. 16). The nucleus is frequently enlarged and occasionally contains a poorly defined basophilic inclusion which, by electron microscopy, is composed of virus particles and fibrillar material (Caras-Cordeo et al., 1981). Although the incidence of HPV infection and the accuracy of diagnosis based on finding koilocytotic change by cytology have not yet been fully documented, the identification of HPV infection of the genital tract is important in preventing sexual transmission of HPV and subsequent development of cervical dysplasia progressing to carcinoma. Women who have genital HPV infection should have follow-up with periodic cytology to detect early atypical changes that may indicate malignant transformation (Davey and Zarbo, 2003).

ASC is a diagnostic category of cells added with the update of the Bethesda System of cytologic diagnosis of cervical Pap smears in 2001 and is designed to indicate cells which may show some, but not all, features of dysplasia or cells of nonneoplastic inflammation change, air drying, or other defects, which may be present in a specimen (Sherman et al., 2004). The ASC realm consists of two major categories, ASC-US, and atypical cells which may be derived from a high-grade squamous intraepithelial lesion (SIL) (ASC-H). ASC-US also includes cells suggesting a low-grade SIL or an indeterminate grade lesion, while the

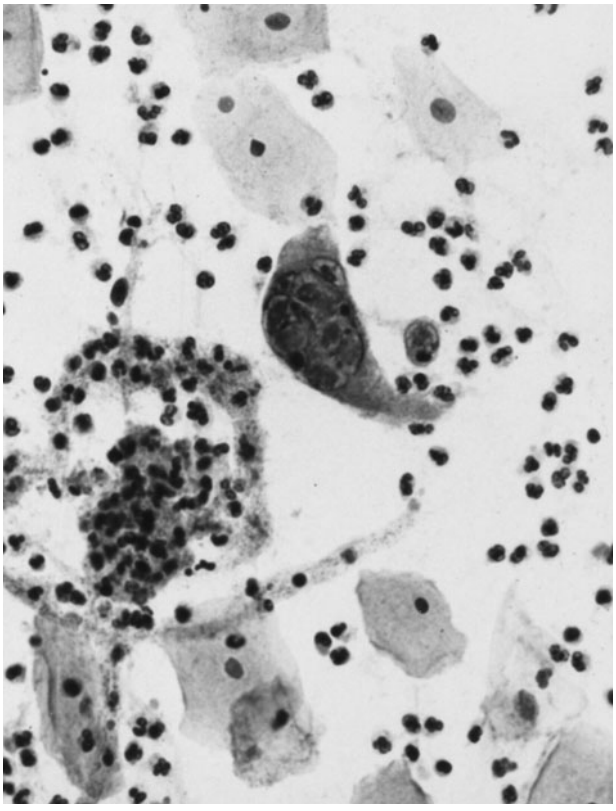

FIGURE 15 HSV in genital smear. Note IN inclusions at different stages of development within one multinucleated epithelial cell. Pap stain. Magnification, ×600.

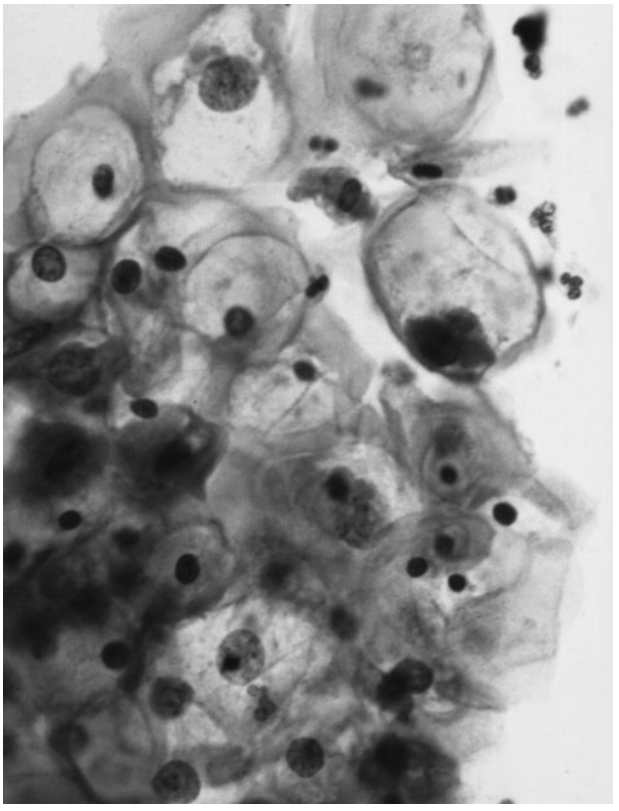

FIGURE 16 Papillomavirus in genital smear. Enlarged squamous cells exhibit koilocytotic changes characteristic of a condylomatous lesion. Pap smear. Magnification, ×800.

designation ASC-H is used to designate only specimens of concern that may come from a high-grade SIL or an underlying cervical intraepithelial lesion designated CIN 2 or 3 (Johnston and Logani, 2007).

The cells in question should show three essential features: (i) squamous differentiation, (ii) an increased nuclear-to-cytoplasmic ratio, and (iii) minimal nuclear hyperchromasia, chromatin clumping, smudging, multinucleation, or irregularity. While the Bethesda 2001 system lists characteristics of ASC cells, it also notes that the ASC category encompasses findings in an entire specimen, not just individual cells.

The importance of the ASC category lies in its clinical follow-up, as indicated by the American Society of Colposcopy and Cervical Pathology in its Consensus Guidelines. Patients with a diagnosis of ASC-US are referred either for repeat cytology in 4 to 6 months or for HPV DNA testing. Patients with either a repeat ASC or SIL on cytology or positive high-risk HPV (types 16 or 18) by molecular testing are referred for colposcopy (Wright et al., 2002). In patients over age 30, HPV testing alone shows promise as an alternative method of monitoring patients, especially those with ASC-H Pap findings.

Because many cervical cytology specimens are now collected using liquid-based methods, samples may be retained for HPV DNA testing if the patient has an ASC result. Because conventional tissue culture isolation of HPV is only a research laboratory method, the virus must currently be detected presumptively by its cytologic changes in Pap smears or with molecular detection of HPV DNA which has proven to be both sensitive and specific (Wu et al., 2006) and is now widely available. The current methods include in situ hybridization, signal amplification, Hybrid Capture II or cleavase, and PCR with detection using either microwell or microarray technology (De Marco et al., 2007).

Other viruses that have been identified by characteristic cytologic findings in vaginal smear include CMV and the poxvirus that causes molluscum contagiosum (Brown et al., 1981). Molluscum contagiosum is a benign cutaneous infection most often observed in children and young adults, which is easily transmitted by direct contact, including sexual transmission. Although the lesions have a characteristic appearance consisting of a small, firm papule with a centralized umbilication on the skin or vaginal mucosa, virus isolation techniques to confirm the diagnosis are not available. However, the histopathology necessitating biopsy and the morphologic changes in individual cells as observed in a Pap-stained smear are diagnostic. The cytologic changes consist of large, dense staining IC inclusions occupying the entire squamous cell and resulting in peripheral displacement of a flattened nucleus (Fig. 17). The cells frequently assume a bean shape with the nucleus displaced to the concave aspect.

Virus Infections of the Eye

In many common ocular lesions, and particularly in those involving the cornea, a rapid diagnosis is essential to initiate therapy and avoid progressive corneal damage. Although biopsy and virus isolation are the usual definitive procedures for the diagnosis of virus infections involving the cornea

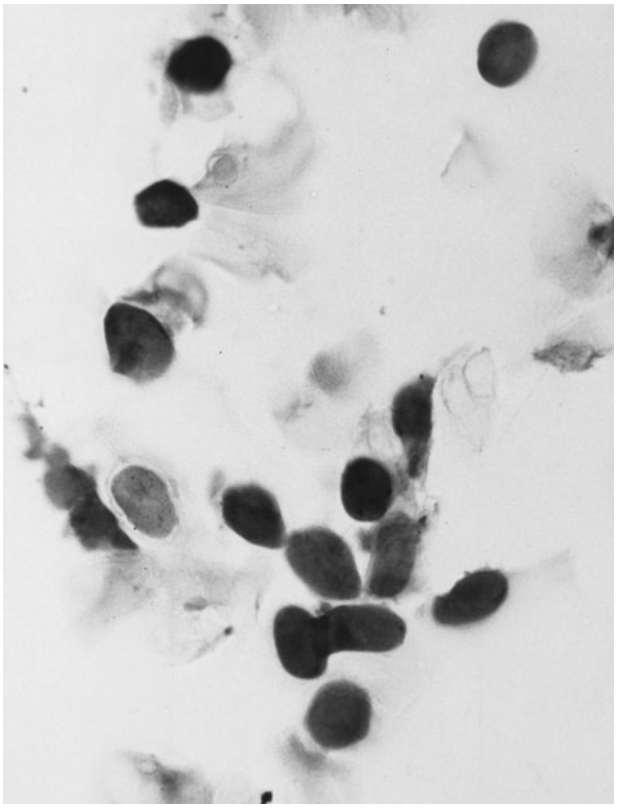

FIGURE 17 Molluscum contagiosum of the genital tract from a skin lesion smear; molluscum bodies showing large IC inclusions displacing and compressing the nucleus. Pap smear. Magnification, ×800.

TABLE 4 Virus and *Chlamydia* infections of the eye

Virus or bacterium	Clinical presentation	Cytologic findings
Adenovirus	Acute (epidemic) keratoconjunctivitis and conjunctivitis with pharyngitis	Multiple eosinophilic IN inclusions (early); dense central basophilic inclusions surrounded by halo (late)
HSV	Corneal vesicle or ulcer; may be isolated ophthalmic lesion or with other HSV vesicles	Multinucleated cells with eosinophilic IN inclusions surrounded by halo; nucleus has ground-glass appearance (early stage)
Molluscum contagiosum virus	Reddish papular 5-mm-diam lesions of eyelid or conjunctiva	Large dense basophilic IC inclusions displacing nucleus
Varicella-zoster virus	Vesicular eruptions in dermatome involving eye (shingles) or accompanying chicken pox	Multinucleated cells with IN eosinophilic inclusions
Chlamydia	Granular conjunctiva with corneal ulcerations (trachoma) or conjunctivitis only (inclusion conjunctivitis)	Enlarged corneal (trachoma only) and conjunctival cells with numerous IC basophilic inclusions surrounded by individual halos

and conjunctiva, exfoliative cytology offers a simple, inexpensive, and rapid means of diagnosing adenovirus keratoconjunctivitis and keratitis due to herpes viruses (Naib et al., 1967; Schumann et al.,1980) (Table 4). Characteristic cytologic changes consisting of multinucleated giant cells in measles keratoconjunctivitis and the large dense IC inclusions of molluscum contagiosum in conjunctival and eyelid lesions may also yield a definitive diagnosis, although these infections as isolated ophthalmic disease are rarely encountered. Finally, cytology is most useful for the diagnosis of chlamydial conjunctivitis, which may be clinically difficult to differentiate from the disease caused by adenovirus but can be diagnosed by the finding of characteristic IC inclusions (Fig. 18) caused by the chlamydial infection pink eye (Gupta et al., 1979).

Specimens containing conjunctival or corneal cells should be collected by a physician, preferably an ophthalmologist, by either a swab or a superficial conjunctival or corneal scraping. Corneal scraping requires examination with a slit lamp microscope for localization of the lesion. The collected material is immediately spread on an alcohol-moistened slide, and after partial evaporation, the slide is placed in 95% EtOH for proper fixation. Because there are usually very few cells and little fluid substrate, air drying of the smears is a frequent problem but can be avoided by immediate fixation of the specimen on the slide.

In adenovirus infections, the conjunctival or corneal cells are mixed with lymphocytes and plasma cells and contain distinctive IN inclusions. In the early stages of infection (Fig. 5), the IN inclusions are multiple, small, and sometimes granular and eosinophilic. At a later stage, the small inclusions coalesce into a single dense basophilic body, usually centrally located and surrounded by a clear halo (Fig. 6).

With herpes keratoconjunctivitis, superficial scrapings from the margin of the ulcerated area will usually contain multinucleated cells with characteristic large eosinophilic IN inclusions surrounded by prominent halos. Early in the infection, as with HSV at other sites, scrapings will reveal enlarged nuclei having a ground-glass appearance.

Herpes zoster keratitis is well recognized clinically and usually does not require additional diagnostic confirmation, such as cytology. However, scrapings of the lesions will yield cells similar to the ones found in HSV infection, although it is reported that syncytia and IN inclusions are less prominent than with HSV (Naib and Elliott, 1967).

The acute conjunctivitis that occurs, usually during the prodrome of measles, is also associated with characteristic cytologic findings seen in conjunctival smears or scrapings. The characteristic giant cells may contain up to 100 round nuclei surrounded by an abundant cytoplasm in which there are numerous eosinophilic inclusions. Occasionally, similar

FIGURE 18 Chlamydia in a conjunctival scraping. Epithelial cells contain IC inclusions surrounded by a clear halo. Pap stain. Magnification, ×800.

eosinophilic inclusions can be observed within the nuclei. These findings can precede the appearance of the typical exanthem by 2 to 3 days.

The morphologic changes in conjunctival and corneal cells due to virus infection must be differentiated from the cytologic changes due to chlamydial infections causing trachoma and inclusion conjunctivitis. In both these diseases, the epithelial cells are generally enlarged and have abundant cytoplasm containing clusters of basophilic IC inclusions, each surrounded by a large individual halo (Fig. 18). In trachoma, the corneal cells are involved, whereas only conjunctival cells show the changes in the more benign chlamydial conjunctivitis. The presence of cells containing IC inclusions in cytologic examination of specimens from the eye suggests chlamydial, rather than viral, infection.

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Electron Microscopy and Immunoelectron Microscopy

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5

In clinical virology, electron microscopy (EM) has achieved a role equivalent to that of conventional light microscopy in clinical microbiology. In both cases, the microorganism is visually detected and its features enhanced by staining. EM allows for the rapid detection of the virus in a clinical specimen, at least at the level of the family into which it is classified, with a very high degree of specificity. However, depending on the concentration and stability of the virus, EM has limited sensitivity compared to other methods of virus diagnosis. While diagnosis at the level of the virus family is frequently clinically meaningful, further identification of the virus at the level of the genus must be accomplished by other methods such as isolation in cell culture or immunospecific or nucleic acid-based approaches.

HISTORY

The ability to visualize viruses had a major impact on the development of virology. The particle one visualizes may only be the relatively inert, metabolically inactive portion of the virus life cycle, but it remains a fundamental feature in the classification and recognition of viruses. Since viruses range from 20 nm to over 300 nm in diameter, their features can only be identified at the nanometer level of resolution, and the optimal instrument to accomplish this is the electron microscope.

Prior to the development of the electron microscope, viruses were considered invisible or ultramicroscopic. Early applications of EM to clinical virology involved distinguishing poxviruses from varicella-zoster virus (van Rooyen and Scott, 1948). The major advances came with the development of simple negative staining technology which allowed for the viral ultrastructure to be visualized (Brenner and Horne, 1959). This opened the door to the application of EM to viral diagnosis and led to unexpected findings especially in the case of gastroenteritis viruses as shown in Fig. 1 and Fig. 2 (Middleton et al., 1977; Petric and Tellier, 2003).

Immunoelectron microscopy (IEM) arose from the combination of EM with the immunospecific interaction of viruses with their respective antibodies (Almeida et al., 1963). Initially, this involved the detection of virus clumps formed when a virus preparation was reacted with its specific antibody. Further developments have resulted in the use of colloidal gold-labeled antibodies to identify virus particles or morphological subviral units (Stannard et al., 1982). Specific antibodies also have been applied to coated grids to selectively enhance the binding of virus particles and thereby enhance the sensitivity and the specificity of the procedure (Gerna et al., 1988).

METHODS

In clinical virology laboratories, the main application of EM is in the direct visualization of the virus in the negatively stained specimen. Conversely, examining viruses in thin-sectioned specimens has been largely assumed by pathology laboratories where virus-like particles are one of a number of markers which are looked for in tissues. Accordingly, the methods discussed below will concern only the direct visualization of viruses after negative staining.

Basic Principles

Diagnostic applications of EM involve a number of discrete steps (Doane and Anderson, 1987). These include the adsorption of viruses to the coated EM grid, the interaction of the viruses with the negative stain, and the recognition of specific morphological features on examination. The copper grid, approximately 3 mm in diameter, is coated with a thin film of Formvar or Parlodion which is further stabilized by coating with a layer of carbon. This surface is able to adsorb virus particles or their subunits as well as other particulate matter in the specimen. It has been shown that viruses present in a drop of water will concentrate over time at the periphery of the drop, and this inherently enriches the quantity of virus in contact with the grid (Johnson and Gregory, 1993).

To be amenable to visualization, the virus must be present at a concentration of 10^6 to 10^7 particles/ml (Doane and Anderson, 1987). The need for such a high concentration can be rationalized if one considers that after accounting for the thickness of the grid bars and the peripheral ring of the grid, the effective area to which viruses can bind and be observed is approximately 3 mm^2. If it is assumed that only the particles present within 10 to 100 μm (100 to 1,000 diameters of a 100-nm-diameter virus) of the grid surface are available to interact through diffusion with the grid coating, then the effective volume that is sampled for examination is between 0.03 to 0.3 μl. If the above assumptions are correct, this represents 30 to 3,000 particles in a specimen

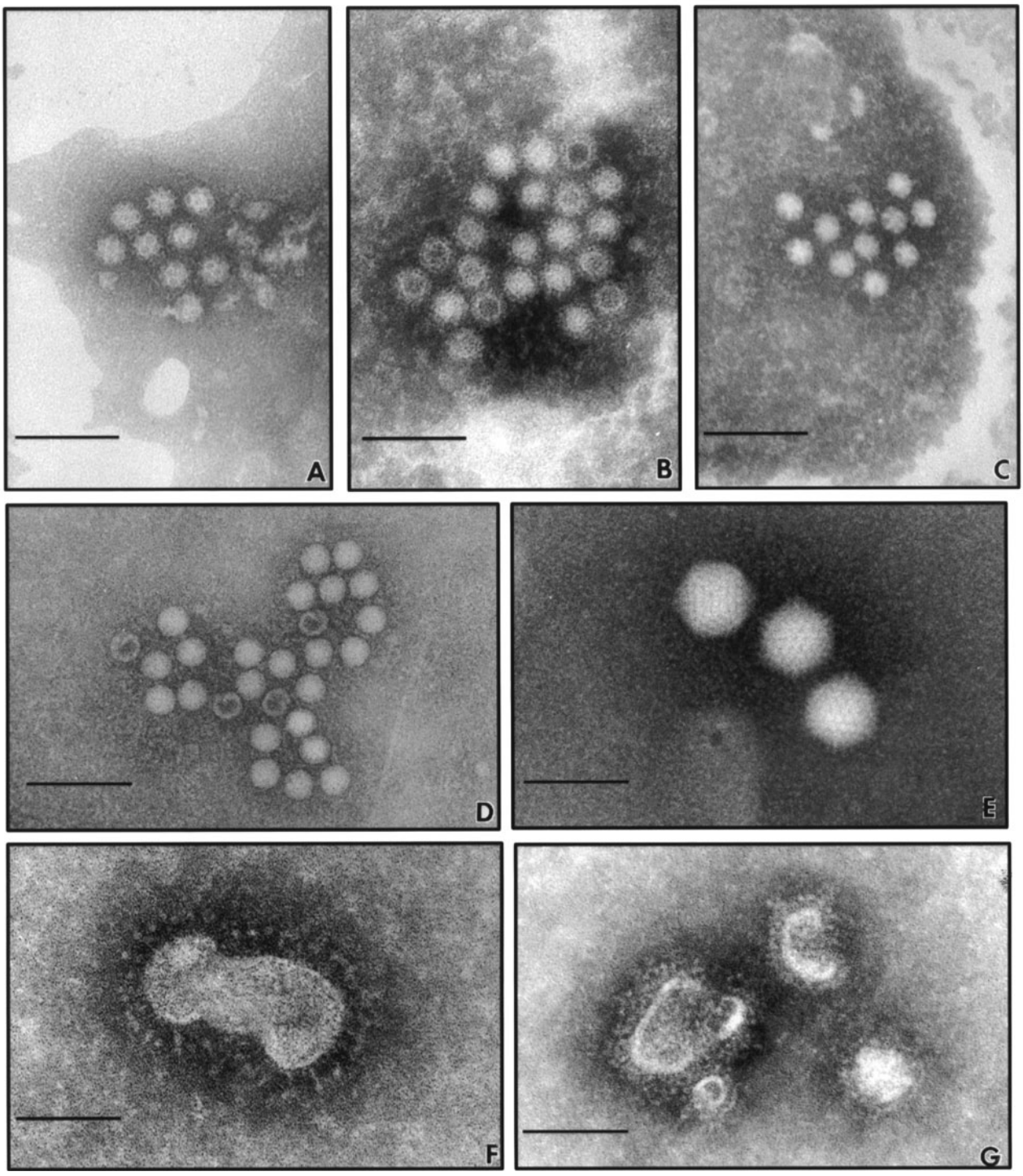

FIGURE 1 Gastroenteritis viruses detected in stool specimens by EM using the direct-application method. (A) Calicivirus; (B) Norwalk-like virus; (C) astrovirus; (D) small round virus; (E) adenovirus; (F) coronavirus; (G) torovirus-like particles. Bars, 100 nm. (Reprinted from Petric and Tellier, 2003, with permission.)

containing 10^6 to 10^7 particles/ml. Only a fraction of these available particles will actually bind to the grid coating and survive the staining process to be visualized. In this context, it can therefore be readily understood why relatively high concentrations of virus in the specimen are needed for diagnosis by EM to be feasible.

Negative staining is based on the principle that the virus particles on the grid will remain unstained, whereas the background will be dark due to the deposit of heavy metal from the stain. Negative contrast is therefore achieved with areas of the grid having limited stain deposit (virus particle) being electron lucent and areas with a more extensive stain

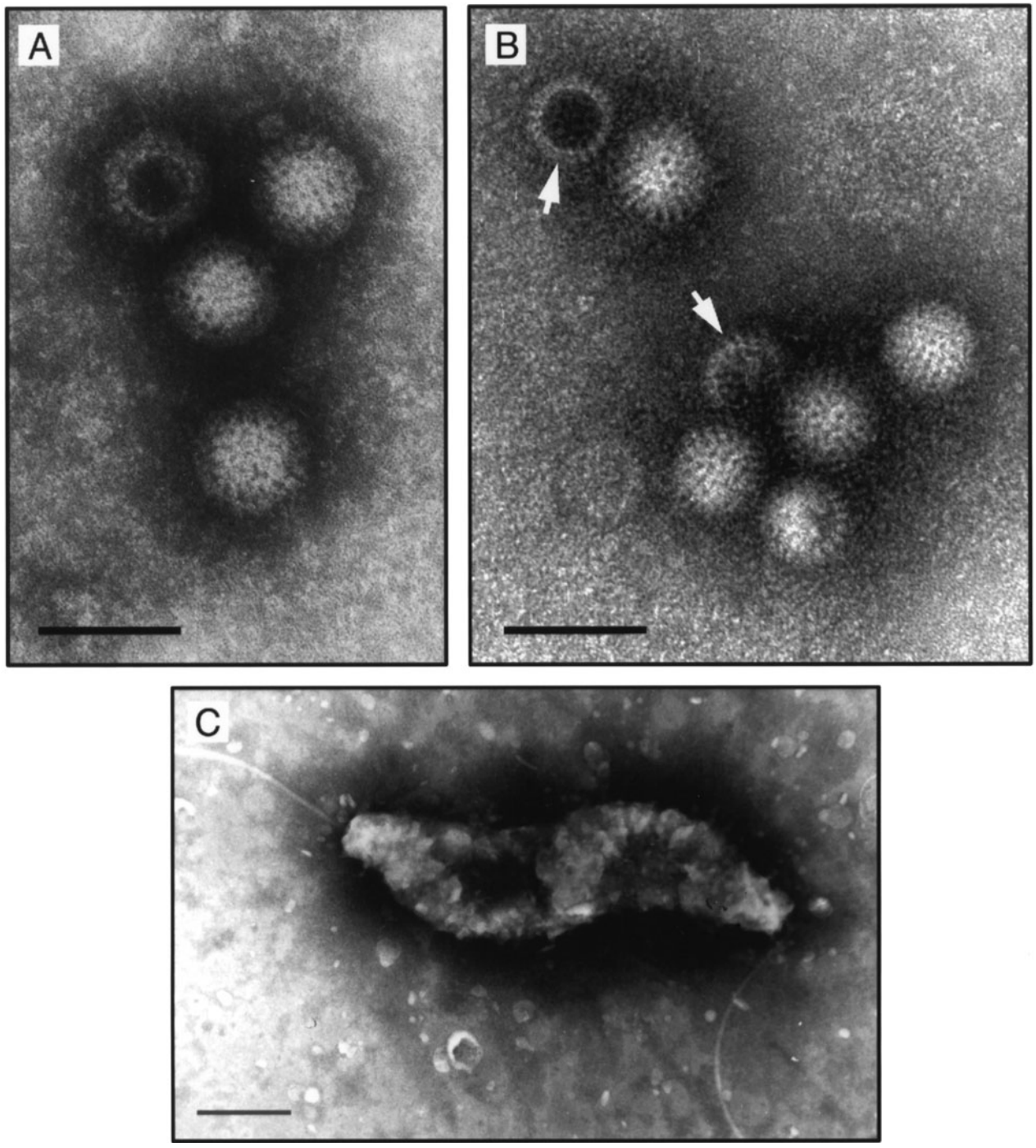

FIGURE 2 Microorganisms diagnosed by EM using the direct-application method. (A) Reovirus; (B) rotavirus. Note differences in arrangement of capsomeres between rotavirus and reovirus. Arrows indicate single capsid particles. (C) *Campylobacter*. Note sinusoidal appearance with bipolar flagella. Bars, 100 nm.

deposit (background) being electron dense. In reality, the virus particles do take up stain both into their interiors as well as into crevices on the virus surface. This is important, since it allows for the visualization of viral capsomeres and peplomers in addition to the size and shape of the virus particles. Stains most commonly used include phosphotungstic acid (PTA) and uranyl acetate (Hayat and Miller, 1990). The former has the formula $PW_{12}O_{40}^{3-}$ and an ionic diameter of 0.8 nm. Uranyl acetate has an ionic diameter of 0.5 nm and is also a weak fixative which stabilizes lipid membranes and is reactive with phosphate and carbonate groups. When using uranyl acetate, the specimens must not contain phosphate buffer, which precipitates and opacifies the visual field. Because of their charged nature, the ions of the stain may not interact with some specimens, and wetting agent such as bovine serum albumin or bacitracin

may be added to the stain solution to overcome this problem (Gregory and Pirie, 1973).

Negatively stained preparations must be examined at a relatively high magnification and at a high level of resolution and contrast. A magnification of ×50,000 is generally sufficient to allow for the recognition of most clinically relevant viruses, and the further 10-fold magnification achieved by examining the image through the binocular magnifier allows for the definitive recognition of most viruses or their subunits. With the application of image capture technologies, the specimen can be examined visually on a high-resolution cathode ray tube or liquid crystal display screen. This can be used to obtain a better resolution of the image at a higher magnification for more precise identification of the virus. For virus diagnosis, the microscope must be aligned for a very high resolution and contrast. Peplomers and capsids require an effective resolution of at least 2 nm to be identified. Similarly, the contrast must be very high in negatively stained specimens, and this is generally achieved with objective lenses of short focal length and appropriate objective apertures. Accordingly, microscopes optimized for thin-section examination may prove suboptimal for virus diagnosis in negatively stained specimens.

Negative Staining Methods

A number of approaches, all using very simple materials, have been described for negative staining of the specimen (reviewed by Doane and Anderson, 1987, and Hayat and Miller, 1990). The copper specimen grids generally used are 200 to 400 mesh. These are coated with Formvar (polyvinyl formal) and stabilized with carbon film. Coated grids can be obtained commercially but are best coated on the premises and can be stored for a prolonged period under vacuum. The negative stain may be a 2% solution of PTA adjusted to a pH of 6.5 with KOH, a 1% solution of uranyl acetate at pH 4.0 or a 3% solution of ammonium molybdate, pH 7.2. The stains are made up in distilled water and sterilized by filtration. To promote its wetting properties, bovine serum albumin may be added to the PTA preparation up to a concentration of 0.05%. The manipulations of the grid are achieved using fine EM forceps adapted to lock in a closed position, a supply of Whatman 1 filter paper for blotting, and a short wavelength ultraviolet lamp for sterilization of the specimens.

Direct-Application Method

The direct-application method is the simplest and most commonly used approach, especially for laboratories where the specimen volume is high. A drop of the specimen is placed on a grid which is held in the forceps. Alternatively, a drop of the specimen may be placed on a glass slide or other disposable surface such as Parafilm and the coated surface of the grid touched to the specimen. The drop is wicked off with the edge of a strip of filter paper, and a drop of the negative stain is added. The stain is then wicked off, and the grid is air dried.

A useful variation of this method has recently been reported in which the coated surface of the grid is altered to bind the virus more effectively (MacRae and Srivasatava, 1998). Prior to the application of the sample, the grid is floated on a drop of 0.1% poly-L-lysine (molecular weight [MW] 35,000) and washed with a drop of distilled water. We have found that this approach enhanced the detection and recognition of gastroenteritis viruses from stools but provided no advantage to the detection of the herpes viruses from lesion aspirates.

Water Drop Method

The direct application is ideal for clinical specimens that have been resuspended in water or a 1% solution of ammonium acetate (Doane et al., 1969). For specimens with a relatively high salt content at physiological or higher levels, the salts tend to precipitate on the grid surface and obscure the viruses present. The water drop method is a rapid approach to remove the excess salt. A drop of sterile distilled water or 1% ammonium acetate solution is placed on a waxed surface such as Parafilm. A small drop of the specimen is then placed on top of the original drop. The coated surface of the grid is touched to the surface of the drop. The grid is then processed for negative staining as described above. Because of the dilution effect inherent in this method, only specimens expected to have a high virus concentration, such as those obtained by density gradient centrifugation or amplification in cell culture, are best processed by this approach.

Agar Diffusion Method

For specimens containing high salt in which the virus is present in a lower concentration, the agar diffusion method has advantages (Anderson and Doane, 1972). Wells of a flexible microtiter plate are filled with approximately 300 μl of 1% agar dissolved in distilled water and can then be stored at 4°C. A coated specimen grid is placed on the surface of the agar, and a drop of the specimen is applied to the grid surface. The specimen drop is allowed to absorb into the agar at room temperature for 30 to 60 min. The grid is removed from the agar and processed for negative staining as described above. This approach is more time-consuming than the water drop method. However, it serves to concentrate the virus in the specimen while removing the excess salt present.

Airfuge Ultracentrifugation

The Airfuge ultracentrifuge has been developed expressly for the centrifugal deposition of viruses in the specimen on the EM grid (Hammond et al., 1981; Hayat and Miller, 1990). Coated specimen grids are placed at the periphery of each sector of the EM-90 rotor. Ninety microliters of the specimen clarified by centrifugation at 10,000 rpm (i.e., a maximum relative centrifugal force of at least 1,456 × *g*) for 10 min are placed in each respective rotor sector. The specimens are centrifuged at 90,000 rpm for 30 min. Grids are removed with forceps, and each grid is inverted briefly onto a drop of negative stain and dried. Decontamination of the rotor is achieved by immersion in a solution of 2.5% glutaraldehyde or 10% formalin and rinsing with ethanol (Doane and Anderson, 1987). Alternatively, a fixed-angle rotor can be used in the Airfuge ultracentrifuge to concentrate the virus. Increased sensitivity of 1,000-fold has been reported, although in our experience this is difficult to achieve. Specimens with a greater volume such as urine may be processed by differential centrifugation, namely, low-speed centrifugation at 10,000 × *g* for 10 min, followed by ultracentrifugation at 100,000 × *g* for 1 h. The pellet is then resuspended in a small volume of 1% ammonium acetate solution and processed for negative staining.

Other methods of processing specimens for EM include the pseudoreplica approach and the use of polyethylene glycol to concentrate the virus in the specimen. The pseudoreplica approach involves the application of a Formvar solution onto the surface of agar on which a specimen has been dried (Lee et al., 1978). The Formvar is then stained with PTA and mounted on a grid. Concentration of virus with polyethylene glycol (MW 6,000) has been used effectively

(Hebert, 1963). While these are elegant approaches for specific viruses and specimens, they do not lend themselves to the routine used in most diagnostic laboratories.

Morphological Features

Viruses are identified at the family or genus level by their distinct size and structure of the virion, the appearance of nucleocapsid or the peplomers of the envelope. Icosahedral viruses ranging from herpes viruses to parvoviruses have nucleocapsids of a defined diameter, and these may also have well-defined capsomeres as shown in Fig. 3A and B. Viruses with helical symmetry may also have well-defined nucleocapsids, such as in the case of the paramyxoviruses as seen in Fig. 4B. These viruses may also have unique peplomers or spike proteins such as in the case of respiratory syncytial virus or influenza virus by which they are can be identified (Fig. 4A and C). For most electron microscopists, the identification of the large viruses such as herpesviruses or poxviruses, shown in Fig. 3A, C, and D, is considered trivial. To identify smaller viruses, the electron microscope must be optimally configured for resolution and contrast. Hence, the differentiation between rotavirus and reovirus (Fig. 2A and B) or norovirus, astrovirus, and sapovirus (Fig. 1B, C, and A, respectively) requires not only knowledge of the respective structures but also a suitably configured electron microscope.

Despite the minimal manipulations of negative staining, some very sensitive viruses do not survive the process intact. Nucleocapsids of viruses which are distinct and stable, such as the herringbone structures of paramyxoviruses (Fig. 4B), may serve to identify the virus. Conversely, viruses such as rubella (Fig. 4F) can only be identified when intact. Such viruses can be stabilized by treating the specimen with 0.5% glutaraldehyde for 30 min at 4°C prior to applying it to the grid (Muller et al., 1983). This stabilizes the virus envelope sufficiently to allow for visualization of the intact particles after negative staining.

APPLICATIONS

Skin Lesions

Vesicular skin eruptions are generally caused by viruses such as herpes simplex or varicella-zoster virus (Fig. 3A). While these infections can be diagnosed with confidence by most health care providers, a laboratory diagnosis is called for in unusual presentations, in immunocompromised patients, or in cases where significant exposure of contacts has occurred, which necessitates broad range intervention, such as the administration of immune serum globulin. Direct examination of the specimen from such lesions can provide a rapid, specific, and relatively sensitive diagnosis. If the lesion is vesicular, the fluid is best aspirated with a 25-gauge needle on a 1-ml syringe. Ideally, the fluid is drawn into the needle without having it enter the syringe barrel. It is then expelled onto a glass slide and allowed to dry. In cases where it is ambiguous whether the lesion is a vesicle or a papule, scraping the surface with the beveled edge of the needle is an effective procedure in that vesicles break and fluid will be visualized, whereas papules remain intact. The fluid is then collected into the needle or picked up by pressing a slide to the exuded droplet. Volumes of fluid obtained by this process range from 1 to 10 µl and prove adequate for diagnosis by EM. The needle may be rinsed by aspiration of medium which is then used for isolation or testing using PCR. Crusted lesions can be lifted with a syringe needle and transferred onto the glass slide with the wet surface touched to the slide. Enough viruses are generally present in the wet part of the crust to achieve a successful electron microscopic diagnosis. Ulcers can be sampled by scraping with the beveled edge of the syringe needle. These may not be as productive. Lesions on mucous membranes such as oral and genital lesions can be collected by aspiration of the vesicle or scraping the ulcer. Such specimens often have fewer viruses, and a diagnosis by EM is more challenging and time-consuming. Suspected herpetic whitlow occurring on the palms may present as a painful whitish discoloration. In collecting such specimens, one should take account of the fact that the skin on the palmar surface is substantially thicker, and more aggressive sampling may be required to reach the vesicle contents. Collection of vesicle or ulcer exudates by swabbing and subsequent transfer to containers containing transport medium has been shown to be suboptimal and is contraindicated for EM diagnosis (Hazelton and Gelderblom, 2003).

Other skin lesions on which a virus diagnosis can be established by EM include the pearly lesions of molluscum contagiosum and vesicles such as those of hand, foot, and mouth disease. The scraping from molluscum lesions may be minimally productive, but the success at visualizing orthopox viruses (Fig. 3C) in such specimens is very high. A presumptive diagnosis of hand, foot, and mouth disease can be made by visualizing small round enteroviruses in aspirates of hand lesions. Lesions of contagious pustular dermatitis or orf are generally large and vesicular. Parapox virus can readily be identified by EM in aspirates of these lesions (Fig. 3D).

In preparing lesion aspirate specimens for negative staining, it is ideal if the specimen is dried on the glass slide and then resuspended in a minimal volume of fluid. This can be accomplished by resuspending the specimen, regardless of its initial volume, in as little as 1 µl of 1% ammonium acetate and touching the preparation with a coated grid. This procedure frequently results in a more concentrated preparation of virus on the grid surface.

Stool Specimens

Viruses are the most common etiologic agents of gastroenteritis, and these include the rotaviruses, caliciviruses, norovirus, sapovirus, astroviruses, adenoviruses, coronaviruses, and possibly toroviruses (Fig. 1 and 2). With this broad spectrum of agents which grow poorly, if at all, in cell culture, comprehensive immunospecific assays have proven difficult to develop. Accordingly, EM continues to have a role in the diagnosis of these agents (reviewed by Petric and Tellier, 2003). This is in part also due to the well-characterized structures of these agents, which are relatively stable to processing, and due to the relatively high concentrations of these viruses in stool specimens, owing to the large surface area of the gut where they replicate.

Stool specimens generally need to be resuspended in either water or a 1% ammonium acetate solution. They may be made as a 10 to 20% suspension, which is then subjected to low-speed centrifugation to clarify the specimen. However, in the event of a high specimen load, a small amount of the stool can be suspended in approximately 10 volumes of a 1% ammonium acetate solution and applied directly to the grid. Centrifugation of the specimen poses the risk that it may remove the immune complexes that form between the virus and the copro-antibody, and it is more time-consuming due to additional manipulations of the specimen.

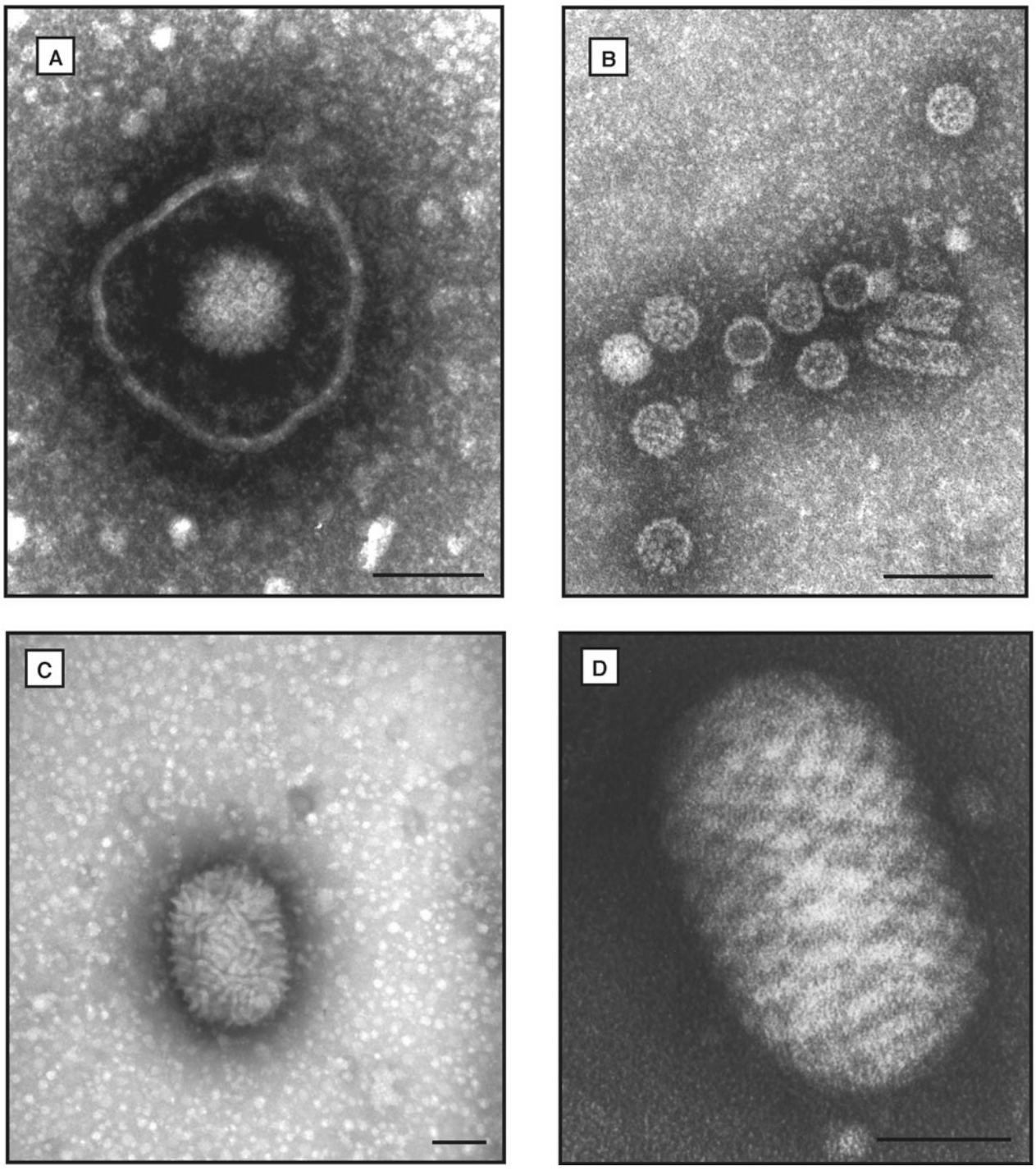

FIGURE 3 Viruses seen by EM in lesion specimens from skin or mucous membrane secretions. (A) Herpes group virus from herpes, varicella, or shingles; (B) papovavirus from respiratory tract secretions of an immunocompromised patient; (C) molluscipoxvirus from molluscum contagiosum; (D) parapoxvirus from orf lesions. Bars, 100 nm.

Given the numbers of etiologic agents involved, the operator must have a thorough knowledge of the virus structures, both as intact virions and as partially degraded particles. For example, rotaviruses can be present as intact smooth particles or as partly degraded particles whose outer shell has been lost (Fig. 2B). Likewise, it is essential that the electron microscope is set at maximum resolution to recognize and differentiate among the astroviruses, noroviruses, and sapoviruses (Fig. 1). Lastly, we have found that *Campylobacter* has a unique sinusoidal morphology with bipolar flagella in characteristic sockets which allows for its presumptive identification by EM (Fig. 2C). Specimens

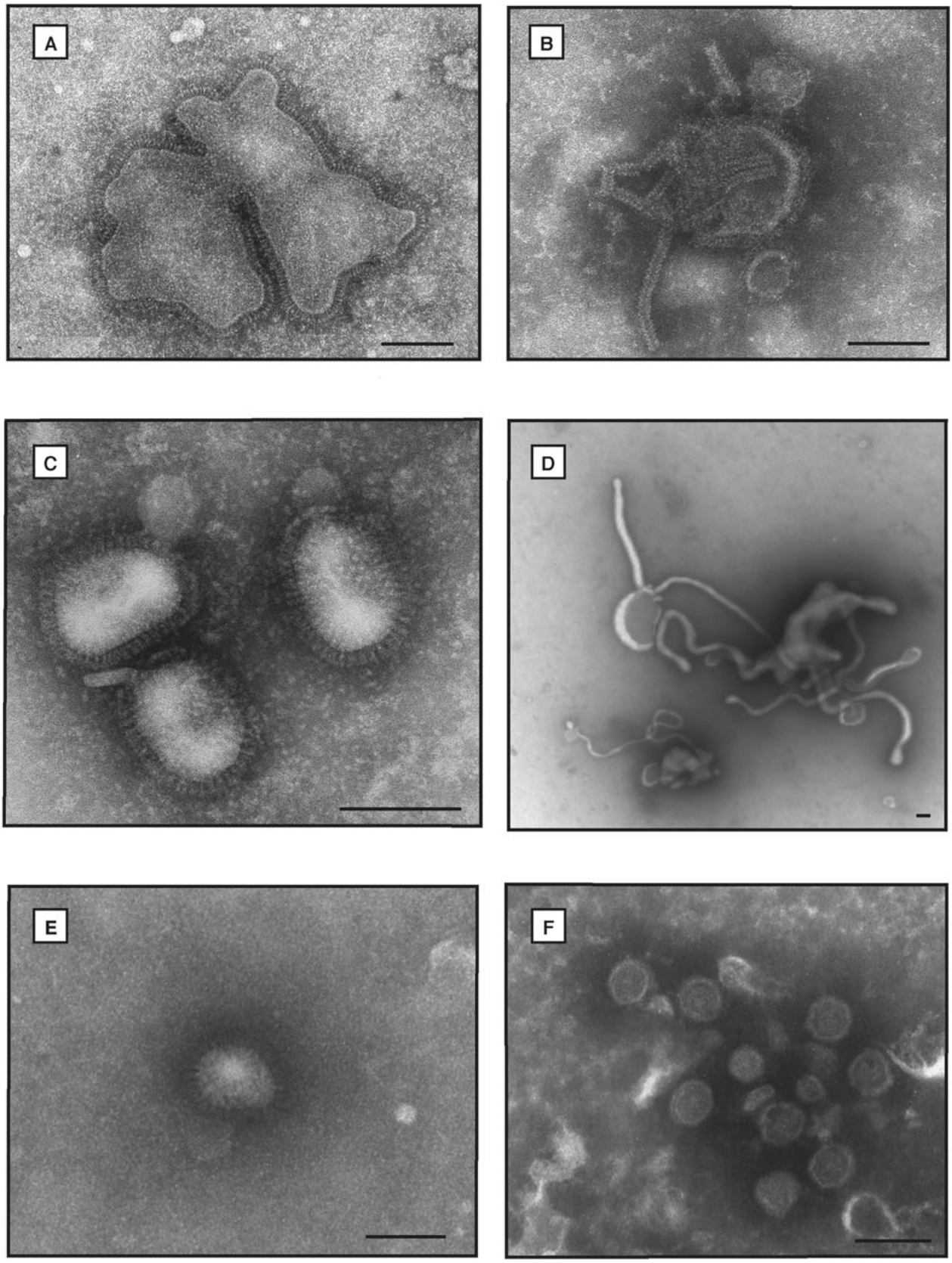

FIGURE 4 Agents seen in EM examination of cell cultures showing CPE. (A) Respiratory syncytial virus. Virus particles are generally intact with well-defined fringe of spikes. (B) Parainfluenza virus. Typically, most particles are broken, with the nucleocapsid visible as herringbone rods. (C) Influenza virus. Note well-defined spike proteins. (D) *Mycoplasma hyorrhinis* from a contaminated cell culture. (E) Foamy agent seen in contaminated cell cultures. (F) Rubella virus from infected cell culture. The preparation was treated with glutaraldehyde to stabilize the viruses. Bars, 100 nm.

containing these structures are then referred for bacteriological work-up.

Urine Specimens

Viruses chronically shed in the urine include cytomegalovirus and human papovaviruses (Howell et al., 1998). These may be present in asymptomatic subjects, and their detection in the urine is difficult to interpret (Lecatsas and Boes, 1980). However, in immunocompromised patients, the diagnosis of these viruses may be relevant. Since adenovirus can cause hemorrhagic cystitis, its detection in the urine is of diagnostic significance. To detect viruses in the urine, it is generally important to concentrate the specimen. The detection of papovaviruses can be enhanced by centrifugation of the urine in a microcentrifuge at 14,000 × *g* for 10 min and examining the pellet by EM. Alternatively, ultracentrifugation at 100,000 × *g* for 1 h will deposit most viruses. However, the resuspended pellets can contain extensive other debris, which may make identification of the viruses difficult.

Other Specimens

Respiratory viruses such as influenza have been detected by EM from respiratory secretions (reviewed by Hayat and Miller, 1990). However, with the ready availability of immunofluorescence microscopy and antigen-based enzyme immunoassays, this approach has become suboptimal. However, we have detected papovaviruses from respiratory tract secretions of patients who suffered from severe combined immunodeficiency (Fig. 3B). Likewise, detection of herpesviruses and paramyxoviruses has been reported in cerebrospinal fluid specimens from patients with complications of varicella and mumps, respectively. Prior to the developments of sensitive immunospecific assays for hepatitis B virus (HBV), the presence of the HBV surface antigen in the blood could be confirmed by IEM. Tissues can be examined for viruses by direct EM. Papillomaviruses from warts and rotaviruses from the autopsy specimens of fatal cases of gastroenteritis can be detected by this approach (Carlson et al., 1978). Examination of other tissues for viruses generally requires fixation and sectioning, and these processes are more effectively performed in pathology laboratories. It must be remembered that viruses in thin-sectioned preparations do not have the same characteristic morphology as those visualized by negative staining.

Identification of Viruses in Cell Culture

EM represents an effective broad spectrum method for examining infected cell cultures for the presence of a virus (Fig. 4). This is feasible, since the limited number of viruses present in the clinical specimens with which the cell culture was inoculated have been amplified to the level at which they can readily be detected by EM. Although identifying the virus present in the inoculated cell culture can be done very effectively by immunofluorescence microscopy after staining the culture with specific monoclonal antibody, EM continues to offer some advantages. With specimens such as stools, either the appropriate antibodies may not be available or the causes of cytopathic effects (CPE) may be diverse and, therefore, the presence of a virus may be readily determined by EM. In other instances, CPE may be relatively nonspecific, and a broad-spectrum process such as EM can identify the virus. A further advantage of examining cell cultures by EM is that contaminating viruses, which cause CPE in primary cell cultures such as the simian paramyxoviruses, mycoplasmas, and foamy agents (Fig. 4D and E) can be readily recognized (Doane and Anderson, 1987).

Cell culture preparations showing CPE are processed for EM by removing and storing the medium and adding 2 to 3 drops of 1% ammonium acetate to the monolayer to resuspend the cells. After a few minutes, the cell preparation is collected by scraping the monolayer, and a portion is applied to the coated grid and processed for negative staining. The cell culture medium may also be examined by applying it directly to the coated grid, or virus suspected to be present may be concentrated by ultracentrifugation as described above. Examination of the medium is preferred in cases where the cell culture has undergone extensive CPE with complete disruption of the monolayer. To reduce the residual salt in the medium, a drop of 1% ammonium acetate is placed on the grid after the cell lysate has been wicked off and removed prior to the application of the negative stain.

IEM

When a virus preparation is mixed with its specific antibody, the viruses or their subunits can be visualized by negative contrast EM as aggregated forms of recognizable viral units. This approach has had a major role in the identification and morphological characterization of new viruses, including rubella, HBV, norovirus, and most recently, human torovirus-like particles, as shown in Fig. 5 (Best et al., 1967; Bayer et al., 1968; Kapikian et al., 1972; Duckmanton et al., 1997).

IEM has been used effectively to increase the sensitivity of virus detection and to serotype a number of viruses (Anderson and Doane, 1973; Edwards et al., 1975; Petrovicova and Juck, 1977). With a defined viral antigen, this approach also has been used to detect seroconversion (Kapikian et al., 1975; Duckmanton et al., 1997).

The detection of virus-antibody interaction by IEM has been greatly improved by the use of antibodies labeled with colloidal gold, as shown in Fig. 5D (Stannard et al., 1982; Beesley and Betts, 1985). This approach can include antibody directly labeled with the gold particles, labeled anti-species antibody, or gold-labeled protein G or protein A, which are available commercially (Geoghegan and Ackerman, 1977). These procedures are sensitive to concentrations of the reagents and require careful titration and adequate controls.

Direct IEM

Various conditions for the reaction of the virus preparation with the antibody have been reported. For stool specimens, a 1:50 dilution of the serum is mixed with a virus preparation, incubated for 2 h at 37°C, and centrifuged at 9,000 × *g* for 15 min. The pellet is resuspended in 1% ammonium acetate, applied to the coated grid, and processed as outlined above. This approach was used in the initial detection of norovirus and hepatitis A virus and the investigations on human torovirus-like particles (Kapikian et al., 1972; Feinstone et al., 1973; Duckmanton et al., 1997). Other variations on this method include incubating the virus with the antiserum for 1 h at 37°C, followed by overnight incubation at 4°C. The mixture is applied to the grid using the agar diffusion method described above (Anderson and Doane, 1972). This is an important step, since the direct application of serum to the grid may result in excess deposits of material, making the recognition of the virus and its subunits difficult.

Serum-in-Agar Method

This approach is based on the principle that antibody in an agar block on which a coated grid has been placed will

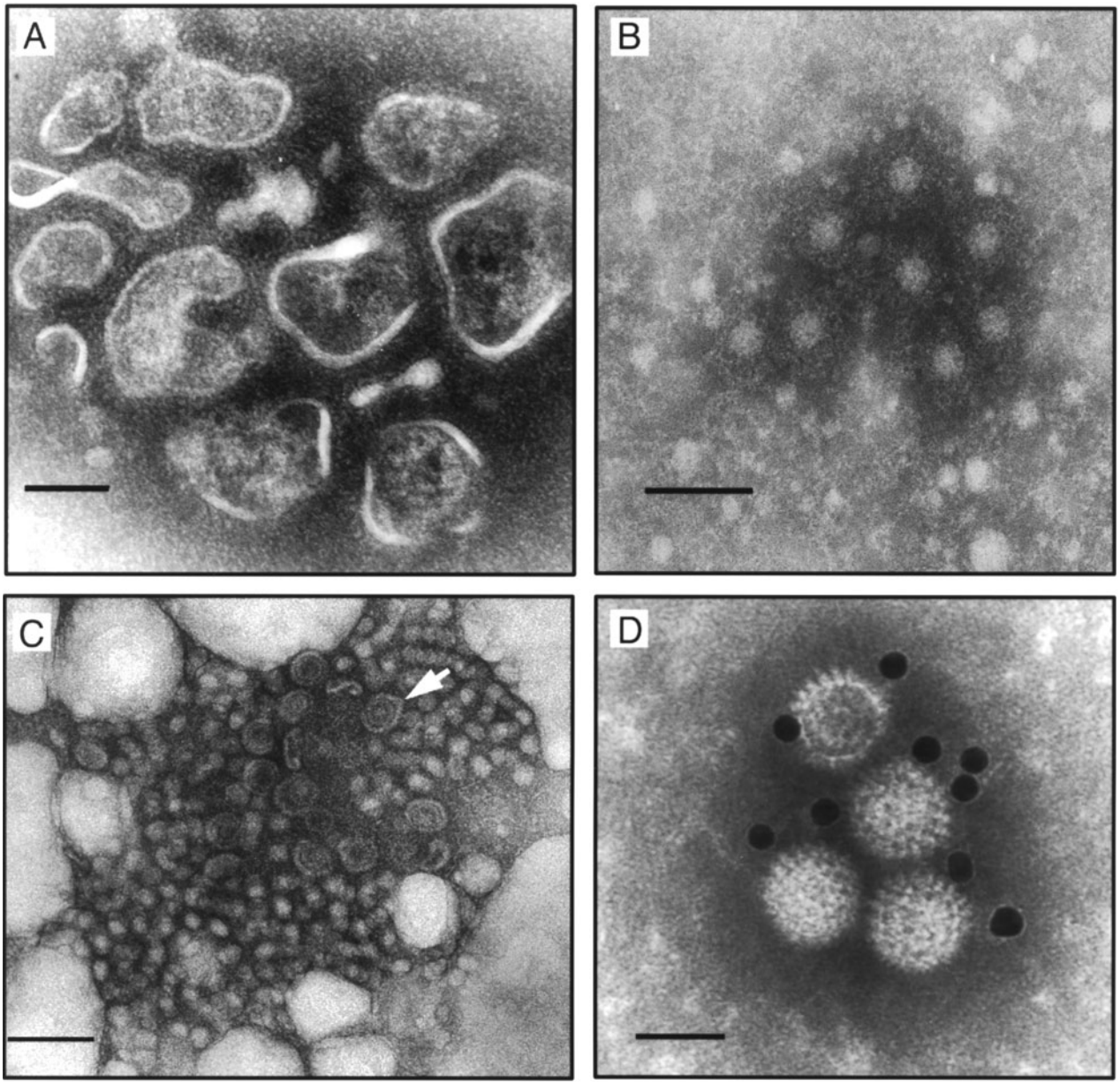

FIGURE 5 IEM of viruses. (A) Human torovirus-like particles reacted with patient convalescent-phase serum. (B) Astrovirus from a stool specimen reacted with antiserum produced in guinea pig. (C) Serum from an HBV-infected patient reacted with reference antiserum to hepatitis B surface antigen. Note intact hepatitis B virions (arrow) and 22-nm-diameter surface antigen spheres. (D) Rotavirus reacted with reference antibody and protein A labeled with colloidal gold. Note association of gold granules with the virus particles. Bars, 100 nm. (Panel D reprinted from Hopley and Doane, 1985, with permission.)

diffuse and react with the specimen to form immune complexes on the grid surface (Anderson and Doane, 1973). A 1% molten agar preparation is cooled to 45 to 50°C, mixed with the desired antibody preparation (serum or monoclonal antibody), and placed into the wells of a microtiter plate as described above for the agar diffusion method. After the agar has solidified, a coated grid is placed on its surface and a drop of the virus specimen is applied to the grid. The drop is allowed to dry at room temperature, and the grid is stained as described for the direct application method above. This has been reported as a very sensitive IEM approach. For optimal performance, the antisera must be used in the optimal concentration range, so titration of these reagents is necessary.

Immunogold EM

A very important development in the diagnosis of viruses by EM was the ability to use antibody conjugated to colloidal gold as an immunospecific marker. This allowed for the definitive identification of virus particles or their subunits based on morphology and immune reactivity, as shown in Fig. 5D. Protein A or protein G labeled with colloidal gold

will react with a wide spectrum of antibodies and can, therefore, be used to identify viruses that have reacted with the antibodies. There are different approaches for carrying out the reaction of the virus with the antibody-colloidal gold conjugate. One common approach involves the adaptation of the serum-in-agar method described above, in which the antibody is incorporated into the agar (Hopley and Doane, 1985; Doane and Anderson, 1987). The virus preparation is mixed with an equal volume of a colloidal gold conjugate of protein A, which is suspended in a buffer of 0.5 M Tris-HCl, pH 7.0, 0.15 M NaCl, and 0.5 M polyethylene glycol (MW 20,000), and the mixture is applied to the grid surface. The virus-conjugate mixture is allowed to dry on the agar surface, and the grid is rinsed in 3 sequential drops of protein A-conjugate buffer, followed by 2 drops of distilled water. The preparation is then subjected to negative staining as described above. The association of the colloidal gold beads of a uniform size with the virus particles or their subunits is indicative of an immunospecific reaction.

Solid-Phase IEM

A fundamental principle of negative-contrast EM is the attachment of the virus to the surface of the coated grid. This attachment can be selectively enhanced by coating the grid surface with an antibody to the virus. This approach has been reported to enhance virus attachment by 10- to 100-fold, thereby improving the sensitivity of the procedure for virus detection by that degree. A further improvement to this approach has been to initially coat the grid with a solution of protein A followed by the specific antibody.

The approach consists of applying a drop of protein A solution to a coated grid (Doane and Anderson, 1987). The grid may be floated on a drop of protein A solution. The grid is then drained and washed by touching it to 3 drops of 0.5 M Tris-HCl buffer, pH 7.2, for a total wash time of 1 to 2 min. The grid is then inverted over a drop of a 1:100 dilution of antiserum for 10 min and again washed by passing over 3 drops of Tris-HCl buffer. The grid is then inverted on a drop of the specimen for 30 min at room temperature, rinsed in Tris-HCl buffer, and stained as described above. This method is useful for detecting a suspected virus present in low quantities in the specimen.

Advantages of EM as a Diagnostic Approach

EM is the most direct approach for virus diagnosis. It has one of the highest degrees of specificity of any diagnostic method, since the virus or its subunits have unique and definitive morphologies. The method provides the most rapid diagnosis of a virus infection for certain specimens, such as lesion aspirates and stools, as well as identification of viruses in inoculated cell cultures showing CPE. Since it is based on virus morphology, it can detect a very wide spectrum of viruses in a single examination, which overcomes the limitations of other assays that are based on a positive or negative response with specific probes or cell lines. IEM allows for increased sensitivity and specificity. It is also allows for the investigation of the immune response to viruses that cannot be cultivated and for which other tests have not yet been developed. From a functional perspective, it has the advantage that it does not require infectious particles and can effectively be performed on appropriately fixed specimens, provided that the viral morphology has remained intact. Finally, in the case of asymptomatic shedding of viruses such as adenoviruses in stool specimens, the lower sensitivity of the electron microscope has an advantage in that it can only detect virus at high concentrations, which is more consistent with a disease process.

EM remains a valuable tool for the primary screening of specimens from emerging infections (Fig. 6) or infections that may be associated with a potential bioterrorist etiology. In the 2003 outbreak of severe acute respiratory syndrome coronavirus (SARS-CoV), EM and IEM were used in examining respiratory specimens directly and after inoculation in cell culture before the etiological agent was identified (Peiris et al., 2003). It was instrumental in the identification of hendraviruses in cell cultures when this virus first emerged (Murray et al., 1995). Finally, it has a well-established potential for the rapid differentiation of varicella-zoster virus from poxviruses in skin lesions (reviewed by Hazelton and Gelderblom, 2003). This technology, therefore, remains an essential component of reference laboratories.

LIMITATIONS

Perhaps the major limitation of EM is the cost and quality of the instrument and its maintenance. This represents a major investment for most institutions, and an electron microscope is often acquired for shared use with pathology or research laboratories. This arrangement may result in the instrument not being readily available for rapid viral diagnosis. It also can lead to the acquisition of an instrument in which the resolution and contrast are the result of a compromise between the requirements of the virology and pathology laboratories, which is therefore suboptimal for observing viruses in negatively stained specimens. The second limitation is the availability of technical staff with a good understanding of virus morphology who have a sound understanding of the process and can attend to minor maintenance matters. The third limitation is the need of an adequate virus concentration in the specimen. Hence, stools and lesion aspirates are acceptable specimens, whereas cerebrospinal fluids and respiratory secretions are generally not acceptable for diagnosis by EM. Specimens such as urine require concentration, which makes the procedure more labor intensive. The final limitation of EM rests in the fact that the specimens must be examined individually, and consequently, the procedure is not open to automation. A qualified operator can only prepare and screen between 30 and 40 specimens per day. The process is therefore ideal for screening relatively few specimens and is not amenable to commercial or other laboratories that process a high volume of specimens.

TIPS

Ideally, the virology laboratory should have its own dedicated electron microscope. While the newest models have excellent features of image processing and alignment, models dating from 30 years ago are still functional and produce excellent images. Hence, an electron microscope is a very long-term investment. To ensure that a microscope is optimally aligned, operating with the appropriate apertures, and providing optimal resolution and contrast to clearly see the viruses and their features, it is valuable to obtain grids with negatively stained preparations of the viruses of interest from a reference laboratory. With these grids, the microscope can be tuned to provide clear images of viruses that can be readily recognized.

For routine diagnostic work, the microscope should have the capacity to readily switch from a low magnification (×500 to ×1,000) to a high magnification (×50,000) suitable

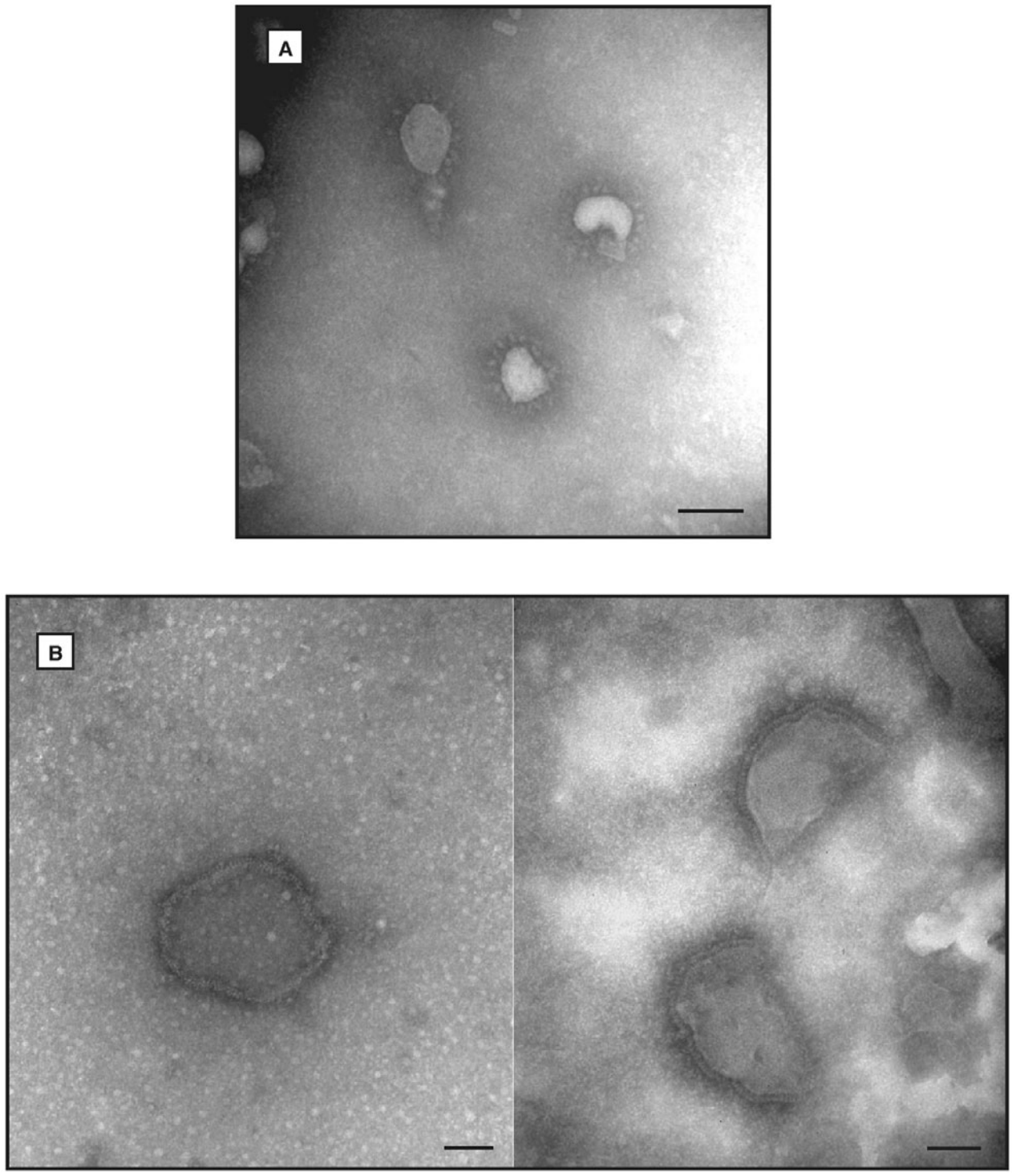

FIGURE 6 Examples of emerging and newly described viruses. (A) Severe acure respiratory syndrome coronavirus from inoculated cell culture. (B) Human metapneumovirus (hMPV). Left panel: hMPV in a nasopharyngeal sample directly examined by EM. The finding of virions by direct sample examination suggests a high viral load. Right panel: hMPV grown in cell culture (R-Mix). Bars, 100 nm.

for virus identification. At low magnification, the grid is scanned to select grid areas that possess an adequate amount of appropriately stained specimen. The instrument is then switched to high magnification to search for virus particles in the selected areas.

It is important that specimens are appropriately collected. Stool specimens in containers are far preferable to rectal swabs, since one is assured that an adequate quantity of material has been collected (Howell et al., 1998). Aspirates of skin lesions are best collected by the virologist or the

designated technologist(s) trained to operate the electron microscope. This ensures consistency in the collection of the specimen and its expeditious processing. It also allows for a decision to be made by experienced professionals as to whether the lesion is suitable for sampling. Since the above procedures involve work with infectious specimens, adequate safety precautions must be employed. The handling of the specimen is preferably done in a laminar flow hood. This may prove challenging when handling stool specimens if the hood is not vented to the outside. A potential safety risk arises when the stained specimen on the grid is removed from the hood and transferred to the electron microscope. Exposure of the grid to an ultraviolet light source of 700 to 1,000 $\mu W/cm^2$ at a distance of 10 to 15 cm for approximately 10 min is considered adequate to inactivate the viruses on its surface (Doane and Anderson, 1987). Similarly, forceps used to handle grids should be dipped into ethanol and flamed immediately after use. With repeated use, metal forceps will become warped. They can be reconditioned by regrinding them. Ceramic forceps are longer lasting in this respect. Grids to be discarded should be either autoclaved or immersed in a 2.5% glutaraldehyde solution.

FUTURE PERSPECTIVES

Computerization has had a major positive impact on the operation of electron microscopes. The developments in image manipulation allow for the display of images on a monitor which further simplifies the recognition of virus particles or their subunits and has major advantages for teaching. Such images can be readily printed, sent to a reference laboratory electronically, or stored for future reference, which greatly facilitates consultation and interpretation. These developments are expected to make EM more user-friendly and reduce the amount of training required to become proficient in this technology.

CONCLUSIONS

The developments in IEM and improvements in instrumentation have greatly enhanced the sensitivity and specificity of diagnostic EM. However, these manipulations are time-consuming; hence, routine diagnostic EM continues to use the simple direct-application method, with refinements being used only on more challenging or unorthodox specimens. With developments of enzyme immunoassays and molecular approaches such as PCR and reverse transcription-PCR, EM is now mainly used in reference laboratories or laboratories in tertiary health care centers. However, in these centers, it remains an indispensable process in the delivery of a comprehensive virus diagnosis.

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Immunofluorescence

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6

HISTORY

The use of fluorochrome-labeled antibody reagents to visualize microbial antigens was first described by Coons et al. (1942). By the 1950s, this technique was used routinely to detect viruses and other infectious agents (Arnaud et al., 1976; Litwin and Grose, 1992) in patient specimens (Llanes-Rodas and Liu, 1956; Biegeleisen et al., 1959; Nichols and McCoumb, 1962; Kirsh and Kissling, 1963; Schaap et al., 1963; Fedova and Zelenkova, 1965; Uchida and Kimura, 1965; Haire, 1969; Baratta et al., 1975; Fulton and Middleton, 1975; Lennette et al., 1976; Olding-Stenkvist and Grandien, 1976; Bikbulatov et al., 1978; Anestad et al., 1983; Grandien and Olding-Stenkvist, 1984) and tissue culture (Gardner and McQuillin, 1968; Lennette et al., 1976; Minnich and Ray, 1980). The early immunofluorescence assays (FA) used noncommercial preparations of polyclonal antisera directed against the target virus and a secondary reagent coupled with either rhodamine or fluorescein. These reagents had significant cross-reactivity due to the presence of antibodies to host cell proteins. Interpretation of results required a great deal of training, skill, and experience. A detailed description of these early methods is provided by Gardner and McQuillin (1974).

In the early 1980s, the use of purified monoclonal antibodies (MAbs) eliminated many of the nonspecific staining problems, resulting in the significant improvement in the quality and consistency of immunofluorescence staining. (Gardner and McQuillin, 1968; Athanasiu, 1985). Over the next few years, reagents containing MAbs directed against herpesviruses and respiratory viruses were described (Showalter et al., 1981; Balachandran et al., 1982; Volpi et al., 1983; Balkovic and Hsiung, 1985; Routledge et al., 1985; Shalit et al., 1985; Swierkosz et al., 1985; Waner et al., 1985; Walls et al., 1986). Although MAbs were more technically difficult to produce than polyclonal antibodies, the increased availability of commercial reagents helped to expand the range of testing offered by clinical virology laboratories. Moreover, many of these reagents were conjugated directly with fluorescein, which shortened the assay to a single step, with a 15- to 30-min incubation, rather than the usual two-step, 60-min indirect fluorescence antibody assay (IFA). This single-step procedure is referred to as the direct fluorescence antibody assay, or DFA. Now, more than 60 years after the first report, immunofluorescence remains one of the primary technologies used by diagnostic virology laboratories (Gallo, 1983; Doing et al., 1998; Barenfanger et al., 2001).

FLUORESCENCE AND THE FLUORESCENCE MICROSCOPE

Fluorescence

Fluorescence is defined as the absorption of a specific wavelength of light by a specific molecule, followed by the nearly instantaneous reemission of light at a different wavelength. Both the excitation and emission wavelengths are defined by the molecular structure of the fluorescent compound, or fluorochrome. When the molecule absorbs light at the excitation frequency, its electrons move to a higher energy state. As the electrons lose energy and return to their ground state, energy is emitted in the form of light. The wavelength of the emitted light is always longer than the excitation wavelength (referred to as "Stokes fluorescence").

A wide variety of fluorochromes have been chemically modified so that they can be directly coupled to proteins. The excitation-emission spectra of one of the most commonly used fluorochromes, fluorescein isothiocyanate (FITC), are shown in Fig. 1. By coupling fluorochromes directly to antibody molecules, these "fluorescent antibodies" can be used as a stain to detect the presence of specific antigens in clinical specimens and virus-infected cell cultures. For this procedure, a fluorescence microscope is required.

Fluorescence Microscopy

A fluorescence microscope is used to permit irradiation of the specimen with light at the desired excitation wavelength and to allow discrimination between that and the weaker emitted light emanating from the specimen. Historically, there are two types of fluorescence microscopes, transmitted light and incident light (epifluorescence). Transmitted-fluorescence microscopes, which are relatively obsolete, are not discussed in this chapter. The three most critical components of the epifluorescence microscope are the light source, the filters, and the objective lenses.

The most often used light sources are high-pressure mercury or halogen vapor lamps, with the former being the most common. Mercury lamps give off high-intensity light in the near-ultraviolet-to-blue range (peak intensities, 313 to 578 nm), the range used to excite the most commonly used fluorochromes in clinical virology, such as fluorescein (490 nm), which gives a green fluorescence, and rhodamine (540 nm), which fluoresces red. In fluorescence microscopy, light from the lamp passes through a barrier filter, which allows only

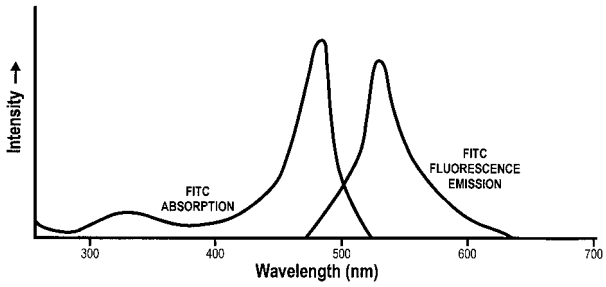

FIGURE 1 Excitation and emission spectra of FITC.

the excitation wavelength to pass (Fig. 2). This light is directed through the objective lens by a dichroic mirror, where it is focused on the specimen. The light emitted from the specimen is collected by the objective lens, travels through the mirror and the second barrier filter (both of which allow light at the emission frequency only to pass) and on to the eyepieces of the microscope. The stained specimen glows brightly against a dark background, because only the emitted wavelength is allowed to reach the eyepieces.

APPLICATIONS

Direct Specimen Testing

A variety of viral agents can be detected directly in various specimen matrices submitted to virology laboratories. Measles, mumps, and herpesviruses (herpes simplex virus [HSV] and varicella-zoster virus [VZV]) are readily detected in patient specimens (Taber et al., 1976; Kumamoto et al., 1988; Waner, 1994). Rabies virus can be detected by immunofluorescence in the brain tissue of infected animals (Gardner and McQuillin, 1980), in skin biopsy specimens taken from the neck and legs of infected individuals, and in corneal smears (Warrell et al., 1988). Table 1 shows the performance characteristics of DFA for several different viruses.

Probably the most widespread use of direct specimen testing by DFA is for the rapid diagnosis of respiratory virus infections. Among those viruses that infect the respiratory tract and can readily be detected in nasopharyngeal exudates are respiratory syncytial virus (RSV), influenza viruses A and B, parainfluenza virus types 1 to 4, adenovirus, and human metapneumovirus (Costello et al., 1993; Waner, 1994; Percivalle et al., 2005). Commercial fluorescence immunoassays are available for the screening of direct specimens for the presence of more than one respiratory virus by using pools of antibodies directed against multiple viruses (Balkovic and Hsiung, 1985; Johnson et al., 1993; McDonald and Quennec, 1993). Several DFA kits are available that contain a screening reagent comprised of MAbs directed against the seven most common respiratory viruses: influenza virus types A and B, parainfluenza virus types 1, 2, and 3, RSV, and adenovirus. Ciliated epithelial cells infected with any of the above viruses react with one or more (in the case of multiple infections) of the antibodies in the pool, giving a positive result. The antibodies in these pools may be directly labeled with one or more fluorochromes (DFA) or have a common fluorescein-conjugated secondary antibody (IFA). To determine with which virus (or occasionally multiple viruses) the patient is infected, the commercial kits contain individual typing reagents specific for each of the above viruses.

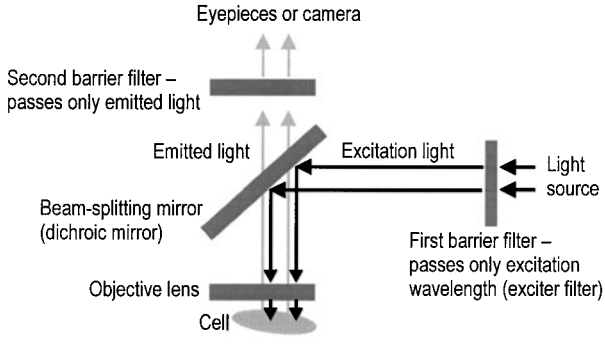

FIGURE 2 Optical system of an epifluorescence upright microscope. See text for details. Courtesy of Douglas Kline, Department of Biological Sciences, Kent State University, Kent, OH.

Several studies have been published describing the sensitivity and specificity of such DFA panels for respiratory virus testing. The results of one such study in a large pediatric population are shown in Table 1. A significant advance in the use of pooled MAbs was the application of multiple fluorescence (Brumback et al., 1993; Brumback and Wade, 1994, 1996). This was first described as a laboratory-developed assay using different reagents for each virus, with multiple staining steps. The reagents were each labeled with a different fluorochrome that emitted blue, green, yellow, or red fluorescence and allowed for the detection of multiple viruses in one well. This staining methodology requires at least two different filter sets to visualize the fluorescence emitted from 400 to 600 μm. Dual-color, "multiplexed" DFAs are now commercially available (Landry and Ferguson, 2000). The first-generation products contain MAbs directed against two or more viruses, with each antibody directly coupled to either FITC or rhodamine. Using the FITC filter set, FITC-stained cells fluoresce with an apple-green color, while those stained with rhodamine appear as yellow-gold to orange. If a rhodamine-tetramethyl rhodamine isocyanate filter set is available, then the apple-green fluorescence disappears and the yellow-gold-staining cells fluoresce a bright, or "hot," pink. Newer commercially available reagents have replaced rhodamine with R-phycoerythrin, to provide a dual-label capability without requiring a change in the filter. Color Plate 4C shows influenza A and B virus-infected cells stained with a single commercial reagent, visualized with a standard FITC filter set. Cells infected with influenza A virus are stained apple-green with the FITC-labeled antibody, while the influenza B virus-infected cells show a yellow-gold fluorescence when stained with the R-phycoerythrin-labeled antibody. The ability to detect two or more different viruses in the same well minimizes the number of slides that need to be prepared and read, can result in significant cost benefits to the laboratory, and improves the turnaround times for reporting of direct specimen results. Some training is necessary for acclimation to reading the yellow-gold stain of dually fluorescent reagents or to differentiate staining patterns of viruses stained with pooled reagents; however, most laboratory personnel become proficient very quickly. These reagents can be used for direct specimen detection or for culture confirmation.

A difference in cellular staining patterns between two viruses can be used to detect and differentiate these viruses in a pooled reagent. An example of such an application is

TABLE 1 Performance characteristics of DFAs versus viral culture

Component	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Complete respiratory DFA[a]	86.7	93.8	87.9	93.1
Adenovirus[a]	62.5	100	100	93.8
Influenza A virus[a]	80	98.6	85	99.6
Influenza B virus[a]	67.4	100	100	98
Parainfluenza virus (1, 2, or 3)[a]	88.5	99.7	96.4	98.9
RSV[a]	93.8	95.6	87	98
HSV (genital)[b]	80	98	92	95
VZV[c]	97.5	60.3	48	97

[a]Adapted from Rocholl et al., 2004.
[b]Adapted from Chan et al., 2001.
[c]Adapted from Coffin and Hodinka, 1995.

demonstrated in Color Plate 4A and B. These cells were infected with either HSV type 1 (HSV-1) (Color Plate 4A) or HSV-2 (Color Plate 4B) and stained with a fluorescent antibody cocktail prepared by mixing HSV-1 and HSV-2 DFA reagents together to detect the cytoplasmic (HSV-1) or nuclear (HSV-2) viral antigens. Because the morphological staining patterns are so clearly different, the two viruses can be easily discriminated.

Direct detection of viruses in clinical specimens has an advantage over culture isolation due to more timely reporting of results. Immunofluorescence has an advantage over enzyme immunoassays, as specimen adequacy can be determined (Balachandran et al., 1982; Lawrence et al., 1984; Ahluwalia et al., 1987; Chomel et al., 1991; Njayou et al., 1991; Takimoto et al., 1991; Ramirez de Arellano et al., 1992; Olsen et al., 1993) and more than one virus can be detected (Grandien and Olding-Stenkvist, 1984; Subbarao et al., 1989; Waner, 1994). Outcome studies have suggested that timely reporting of viral infections, as determined by direct specimen testing, can result in decreased mortality, decreased length of hospitalization, and better antibiotic stewardship (Woo et al., 1997; Barenfanger et al., 2001).

While the specificity of direct specimen detection is generally excellent, sensitivity is often low compared to culture, except for labile viruses such as RSV and VZV (Minnich and Ray, 1980; Schmidt et al., 1980; Douglas, 1985; Solomon, 1988; Takimoto et al., 1991; Thomas and Book, 1991; Dahl et al., 1997; Freymuth et al., 1997). The use of cytocentrifugation, rather than drop suspension, to prepare slides has been shown to improve sensitivity for the detection of HSV (Landry et al., 1997a, 1997b), and many laboratories are now using cytocentrifuged slides for all direct specimen testing (Landry et al., 1997a, 1997b; Doing et al., 1998). However, direct testing of patient specimens should not be relied on as the only method of virus detection; culture is recommended for all specimens that are negative by direct specimen testing.

Immunofluorescence is the preferred method for the detection of the 65-kDa lower matrix protein of cytomegalovirus (CMV), phosphoprotein 65 (pp65), in peripheral blood leukocytes of patients at high risk for severe CMV disease. It is not yet clear if the presence of the antigen reflects infection of leukocytes, phagocytosis, or both. However, an increase in the number of positive leukocytes has been seen in patients with active clinical disease (Color Plate 4E). The antigenemia assay has been shown to be a rapid, sensitive, and specific method to predict and differentiate CMV disease from asymptomatic infection in organ transplant patients and persons with AIDS. Immunofluorescence is not the first test of choice for detection of viruses in tissue samples, as the structural morphology of the tissue cannot be readily discerned.

To detect CMV pp65 antigen, slides are prepared from peripheral blood leukocytes isolated from anticoagulated blood (sodium citrate, sodium heparin, EDTA, or acid citrate dextrose are acceptable) by cytocentrifugation (Storch et al., 1994; Landry et al., 1997a). The leukocytes may be isolated by using dextran sedimentation or other separation methods or by lysis of red cells by ammonium chloride (Ho et al., 1998). Since the CMV antigenemia assay is quantitative, the cells must be counted prior to cytocentrifugation; typically 2×10^5 leukocytes are used to prepare each cell spot. For optimum sensitivity, studies have shown that the samples should be processed within 8 h of collection, as there can be a loss of positive cells beyond this time (Landry et al., 1995). Positive cells can still be detected in anticoagulated blood that is several days old (Brumback et al., 1997), but quantitation is not accurate. Formalin is superior to acetone for fixation (Perez et al., 1995); the fixed cells are permeabilized with a nonionic detergent such as NP-40 and stained. Positively stained cells show typical staining of the nucleus (Color Plate 4E). Results are expressed as the number of positive nuclei per 2×10^5 leukocytes. The significance of the result depends on clinical diagnosis, patient history, symptoms, and results of other tests, including previous CMV pp65 results. Commercial kits containing the reagents needed to perform the antigenemia assay are available.

Standard Culture

Cell culture is the gold standard for virus detection and diagnosis. As virus is amplified by growth in cells, it offers the advantage of sensitivity over direct specimen testing, except for fragile viruses, as previously indicated. The major disadvantage of culture is the relatively slow turnaround time: anywhere from 7 to 28 days depending on the virus.

Each virus, or family of viruses, has a distinct cytopathic effect in culture that can be used for presumptive identification. Presumptive identification of ortho- and paramyxoviruses can also be made by adsorption of blood cells (hemadsorption) to infected cells. Definitive identification is then confirmed by immunofluorescence.

Commercial FA reagents are available for the majority of cultivable viruses, including RSV, parainfluenza virus types

1 to 4, influenza viruses A and B, measles and mumps viruses, HSV-1 and -2, CMV, VZV, adenovirus, human metapneumovirus, and many of the enteroviruses. These reagents include those that identify individual viruses (CMV, HSV, and RSV, etc.), antibody pools directed against several viruses (as for the common respiratory viruses), or those with broad group identification (pan-enterovirus reagents). Both IFA and DFA reagents are available.

Shell Vials

The spin-amplified shell vial assay has significantly shortened the turnaround time for virus identification in cell culture, particularly for those viruses, such as CMV, that take a long time to grow (Gleaves et al., 1984).

Shell vials are 1-dram glass vials containing a coverslip, upon which cell monolayers are grown. Vials with subconfluent monolayers (particularly for CMV) are preferred. Specimens are inoculated directly into the vial and centrifuged at 25 to 37°C at 700 × *g* for 40 min. Higher *g* forces for shorter time periods also have been shown to be effective (Engler and Selepak, 1994). Maintenance medium is added to the vials, and they are incubated at 37°C for 18 h to 4 or 5 days.

After the incubation period, the maintenance medium is removed; the coverslips are washed with phosphate-buffered saline (PBS) and fixed with acetone. The coverslips can then be stained directly in the shell vial or removed and mounted on a microscope slide for staining (Clarke, 1998).

Shell vial cultures of MRC-5 cells are routinely used for CMV. Other cells, such as RMK and A549 cells, etc., have been used successfully to isolate other viruses. Many viruses, such as CMV and other herpesviruses, can be detected within 24 to 48 h, before the development of cytopathic effect; others may require up to 5 days before detection (Engler and Preuss, 1997). Depending on the virus, different cell lines and combinations of multiple or mixtures of cell lines can be used in shell vial culture systems. Respiratory viruses (Matthey et al., 1992), CMV, VZV, rubella virus, enteroviruses, HSV, human herpesvirus 6, and human herpesvirus 7 have all been detected in shell vial cultures. More recently, mixtures of different cells within the same vial have been made commercially available. One such product permits the detection of seven of the most common respiratory viruses in a single shell vial (RSV, influenza virus types A and B, parainfluenza virus types 1 to 3, and adenovirus) (Barenfanger et al., 2001; St. George et al., 2002). This first generation product consisted of both mink lung and human lung carcinoma cell lines, Mv1Lu, and A-549 respectively. A newer mixed-cell product substituted MDCK cells for the Mv1Lu, permitting detection of human metapneumovirus in addition to the seven viruses mentioned above. Similar mixed-cell shell vials also are available, one for the rapid, single shell vial detection of VZV, HSV, and CMV (Huang et al., 2002a), and another for the detection of a broad range of enteroviruses (Huang et al., 2002b). The latter contain a cell line that has been genetically modified to enhance their sensitivity to infection by enteroviruses.

Specimen Collection and Processing

Proper collection and transport of specimens is critical for successful detection of viral antigens by FA. Specimens that are considered most appropriate for analysis by immunofluorescence include nasopharyngeal swabs, aspirates, or washes, bronchoalveolar lavage samples, swabs (including the recently introduced flocked swab specimen processing technology) or scrapings from vesicular lesions, tissue biopsy specimens (e.g., lung, liver, and brain), blood leukocytes, conjunctival cells, corneal scrapings, and urine sediment. The use of flocked swabs, rather than the traditional Dacron swab, has resulted in a significant improvement in the recovery of cells from a variety of specimen types, resulting in increased sensitivity of DFAs and decreased specimen rejection due to insufficient numbers of cells in the sample (Daly et al., 2006; Barger et al., 2006). Specimens that should be considered inappropriate are throat swabs and gargles, which contain very small amounts of virus-infected cells. A comprehensive discussion of the selection, collection, transport, and processing of specimens can be found in a separate chapter. A brief discussion of how various types of specimens are processed for FA testing is found below.

Nasopharyngeal Aspirates and Washes, Tissue Aspirates, and Swabs Submitted in Viral Transport Medium

These specimens require centrifugation to collect cells for FA analysis. When swab specimens are submitted in viral transport medium, thoroughly mix the sample on a vortex mixer and firmly press the swab against the inside of the transport tube to express all collected cells, virus, and fluid. Centrifuge the specimen at 600 × *g* for 5 min to pellet the cells. The cell pellet is suspended with 5 ml of sterile PBS at a pH of 7.0 to 7.6 and centrifuged as described above. All but 100 to 200 µl of the PBS is removed; a uniform suspension of cells in the remaining fluid is made by gently pipetting up and down. A drop of the cell suspension is placed into one or more wells of a Teflon-coated multiwell glass slide and air dried. The slide may be placed on a slide warmer to facilitate drying, but care must be taken to avoid excessively high temperatures that may denature the viral antigens, resulting in loss of reactivity with the antibody. Alternatively, methods for preparing slides from cell suspensions by cytocentrifugation have been reported previously (Landry et al., 1997a, 1997b; Doing et al., 1998). Cell spots prepared with a cytocentrifuge were found to be more uniform than those prepared by the method described above and had less nonspecific staining, as mucus and other debris were centrifuged out of the well; also, they were easier to interpret by personnel with less training and experience. An additional benefit is that the smaller cell spot required less volume of reagent to stain the cells, resulting in cost savings for the laboratory.

Preparation of Slides Directly from Swabs

It is often expedient to make a slide from a swabbed clinical specimen immediately after the sample is taken. This is usually the case with swabs obtained from vesicular lesions caused by HSV, VZV, or enterovirus infections. To prepare these slides, a fresh vesicular lesion should be chosen and opened with a sterile scalpel. The base of the vesicle is swabbed firmly with a cotton or Dacron (not calcium alginate) swab. The swab is then rolled (not rubbed) onto the appropriate number of wells of a Teflon-coated glass slide. (Rubbing the swab onto the slide may result in damage to the cells, making the slide difficult to read.) Slides prepared in this manner should be air dried completely before fixation.

Fixation

Prior to staining, the specimen must be properly fixed. The most commonly used fixative is cold acetone. Acetone absorbs moisture if it is not properly stored. Moisture causes a nonspecific hazing during the fixation process; therefore, it is important to ensure that fresh acetone is used for fixing the specimens. Acetone that appears to be cloudy should be

discarded. The time of fixation of specimens does not appear to be overly important; 2 to 10 min is more than adequate for this purpose. It also is possible to flood the slide with acetone and allow it to evaporate. However, the majority of commercial kits have validated their reagents with slides fixed for 10 min, and changing the fixation procedure may adversely affect staining. Many high-volume laboratories have replaced the traditional 1-dram vial used for shell vial assays with plastic multiple-well cell culture plates. In this case, a mixture of methanol-acetone or ethanol-acetone must be used as a fixative, since polystyrene is not compatible with 100% acetone. Methanol alone as a fixative is not compatible with many FA reagents; water is not recommended as a diluent in place of methanol or ethanol.

PRACTICAL DETAILS FOR DIRECT AND INDIRECT IMMUNOFLUORESCENCE STAINING

Historically, most immunofluorescence staining procedures were indirect; that is, the specimen was initially reacted with a primary antigen-specific antibody reagent, binding of which is detected using a secondary antibody-fluorochrome conjugate directed against the primary antibody (Fig. 3A). This procedure is referred to as the IFA method. Today, most immunofluorescent antibody assays are performed using a single antibody directly conjugated to a fluorochrome such as FITC. A significant advantage of using directly labeled antibodies for antigen detection is speed; by eliminating the second staining step, the assay time is virtually cut in half or better. The use of directly coupled antibodies is referred to as the DFA (Fig. 3B). A downside of the DFA procedure is that the intensity of fluorescence may be lower than with IFA. Commercial reagents may compensate for the lower intensity by using a cocktail of two or more antibodies that react with different antigens or different epitopes on the same antigen; this results in a greater number of labeled antibodies binding to each infected cell and thus in increased intensity of fluorescence. Most commercially available DFA reagents or labeled secondary antibodies contain a counterstain such as Evans blue to allow nonfluorescing cells to be visualized. Evans blue-stained cells fluoresce red when visualized through a fluorescence microscope, making it easier for the operator to focus on the specimen and to determine the adequacy of the specimen by the number and morphology of cells present in the well.

The following procedures should be used as a guide for performing both DFAs and IFAs:

1. During the staining process, slides should be placed in a humidified chamber to prevent evaporation of reagents. Several types of humidified chambers are available commercially; alternatively, a closed box with a wet sponge or moistened paper towels will suffice.

2. The specimen is completely overlaid with the primary antibody, after which the slide is placed in the humidified chamber and incubated at 37°C. The length of incubation

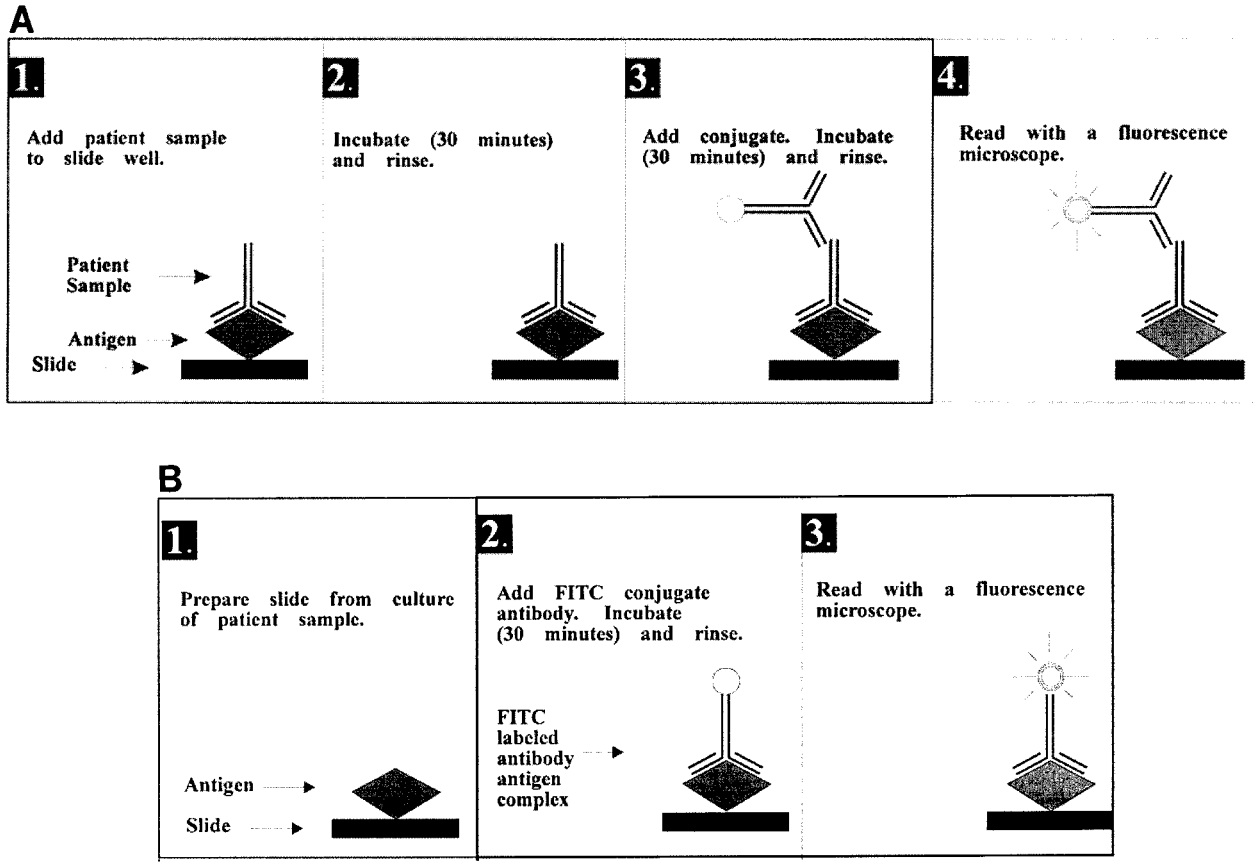

FIGURE 3 Schematic representation of IFA (A) and DFA (B).

is variable, based on the antibody concentration and its affinity for the antigen. Most commercial immunofluorescence kits suggest a 15 to 30 min incubation.

3. After staining, the primary antibody must be removed by washing. Immersion in a Coplin jar containing fresh PBS is the most gentle and efficient method. The addition of Tween 20 to the buffer can help to remove any nonspecific antibody binding. A series of one or two washes of 1-min duration is sufficient if the primary antibody is a MAb. Polyclonal antibodies may require longer washing. Overwashing is undesirable, since it may lead to diminished staining and false-negative results. Washing conditions need to be established for each different antibody being used. If the primary antibody is directly coupled to the fluorochrome (DFA), skip to step 7.

4. Remove excess PBS from the slide, being careful not to touch the areas of the slide containing the specimen.

5. Overlay the specimen with the labeled secondary antibody and incubate as described above.

6. Wash the slide as described in step 3.

7. If PBS is used for washing, the slides should be mounted immediately. Excess PBS should be blotted or shaken from the slide, again taking care not to disturb the specimen. If the slides are to be air dried before mounting, rinse in distilled water and then dry completely. A drop of mounting medium is placed on the specimen area followed by a glass coverslip. Mounting medium, usually supplied with commercial kits, contains buffered glycerol, pH 9.0, with photobleach inhibitors to prolong fluorescence.

Reading and interpretation of results for DFA and IFA require critical evaluation to ensure reliable results. Staining patterns are highly dependent on the type of virus being detected as well as on the specificity of the primary antibody being used. The control slides supplied with commercial kits may be used as an indicator of expected results. Direct smear preparations can contain fragments of disrupted cells as well as leukocytes, both of which can trap fluorescein and result in staining artifacts, making the slide difficult to interpret. Contaminating bacteria, such as *Staphylococcus aureus*, can bind the Fc portion of antibodies and may cause false-positive results. Specimen quality can be verified by the number and type of cells present on the slide. For example, good nasopharyngeal specimens should have at least three columnar epithelial cells per high-power field (magnification, ×400). Antigen-negative specimens that contain fewer cells or that contain a large number of squamous epithelial cells should be considered poor quality, with suspect results. Each laboratory must develop its own criteria for interpreting slides as positive and negative, based on staining patterns and intensity of fluorescence.

TROUBLESHOOTING

False-positive results can be due to several causes, such as nonspecific staining due to binding between the Fc region of the antibody and protein A or G of *S. aureus*, a contaminant in some respiratory specimens. Inflammatory cells such as lymphocytes and polymorphonuclear leukocytes contain Fc receptors, and herpesvirus-infected cells (including CMV, HSV, and VZV) (Keller et al., 1976; Litwin and Grose, 1992; MacCormac and Grundy, 1996) also express Fc receptors that might cause nonspecific binding of antibodies. False-negative results can arise from a variety of causes; the most common are described in Table 2. The troubleshooting guide in Table 2 describes commonly encountered problems with FA and suggests ways of resolving them (Clarke, 1998).

QUALITY ASSURANCE/QUALITY CONTROL (QC)

The majority of FA reagents currently used by clinical laboratories are purchased from commercial sources. These reagents fall under the jurisdiction of the U.S. Food and Drug Administration (FDA), which, at minimum, requires that they be manufactured under "current good manufacturing practices" to ensure consistent quality. Many kits also require premarket approval [510(k) clearance] before they can be used for diagnosis. Consequently, performance characteristics, such as sensitivity, specificity, cross-reactivity, and prevalence, etc., have been established for these reagents and are included in package inserts. Validation of commercial reagents usually requires two to three clinical sites with a minimum of 200 specimens per site, depending on the prevalence of the virus.

The critical components for laboratory-developed assays are now regulated by the FDA and must be labeled "Analyte Specific Reagent" by the manufacturer. Their sale is restricted to Clinical Laboratory Information Act high-complexity labs, public health labs, Veterans Administration hospitals, and manufacturers. These assays also must be validated before the laboratory can use them. Clinical Laboratory International Standards should be followed for reagent and assay validation.

Quality Assurance

Quality assurance establishes standard operating procedures for all aspects of testing, including specimen collection and processing, assay protocols, validation requirements, and QC for all tests performed in a laboratory. Critical components, such as swabs, buffers, and transport media, etc., should also be included, along with package inserts from the commercial kits used in the laboratory. If laboratory-developed assays are used, recipes, procedures, and QC specifications should be included. Package inserts should be read carefully, with close attention paid to the intended use of the kit, assay procedures, QC, performance characteristics, and limitations of the assay. Any deviation from manufacturers' recommended procedures must be validated by the laboratory.

Standard operating procedures are living documents and should be reviewed and updated regularly to ensure that they reflect actual practices in the laboratory.

QC

QC refers to those controls that are run at established intervals to ensure that procedures and reagents (including cells) are behaving appropriately. Commercial kits usually include control slides to be used along with direct specimen testing and to act as a control for the reagent. For culture confirmation, known positive and negative isolates should be inoculated into cell culture to ensure proper cell culture technique, slide preparation, and staining. Previously isolated viruses can be used as controls, or known strains may be purchased from commercial sources such as the American Type Culture Collection. QC specimens should always be tested first to confirm that the assay has worked correctly. If the controls fail, the assay is considered to have failed as well.

TABLE 2 Problems most frequently encountered with FA[a]

Problem	Cause(s)	Solution
Weak or no specific fluorescence (including positive controls)	Wrong (too weak) concentration of immunoreagent	Retitrate reagent
	Deterioration of reagent	Retitrate and/or replace; store properly, and aliquot if necessary
	Counterstain too strong	Review concentration and counterstaining time
	Rapid fading of fluorescence	pH of mounting medium must be greater than that of wash buffer; use mounting medium that contains photobleaching inhibitor
	Wrong filters or inadequate light source	Review use and maintenance of equipment; replace light bulb
	Cannot achieve sharp focus because of dirty or improperly focused optics	Review procedures for maintenance and use of optics
	Nonspecific glare or haze masking specific fluorescence	Allow cell spots to air dry completely before mounting; use fresh acetone
	Antigens destroyed by fixative	Do not use fixatives other than acetone without determining effect on antigen stability
Weak or no fluorescence with test slides but positive controls acceptable	Controls do not adequately reflect actual test conditions	Review procedure; determine whether same or different cells, reagents, or methods were used for test and control slides
	Improper slide cleaning, preparation, or fixation	Review procedures
Nonspecific fluorescence and/or false positives with test slides and negative controls	Wrong concentration (too strong) of immunoreagent used	Retitrate reagent
	Cross-reactive immunoreagent	Identify non-cross-reactive concentration of reagent, or obtain a better product
	Binding of antibody via Fc receptors	Use conjugated $F(ab')_2$ fragments
	Autofluorescence of cells	Use alternative cell line, or incorporate counterstain for better resolution
	Trapping of immunoreagent on heavy or raised preparations	Avoid heavy cell preparations which obscure cellular morphology and complicate reading; increase number of washes during staining
	Inappropriate immersion oil	Use immersion oil designated for fluorescence microscopy
	Inadequate removal of fixative	Air dry cell spots completely after fixing; rinse slides with PBS and/or water just prior to staining
	Water in acetone	Replace acetone
	Slides dried during staining	Use properly sealed moist chamber during incubation; apply adequate reagent volumes to cell spots
	Mounting medium not appropriate for immunofluorescence, or pH too low	Obtain proper reagents; pH of mounting medium must be greater than that of wash buffer
	Mounting medium applied to wet cells	Allow cell spots to air dry completely before mounting
	Wrong filters or equipment	Review use and maintenance of equipment
	Reading error	Must be able to differentiate specific patterns of fluorescence from nonspecific staining
	Precipitated material in immunoreagent	Microcentrifuge reagent for 1 min, or filter through a 0.45-μm-pore-size filter
Nonspecific fluorescence and/or false positives with test slides but negative controls acceptable	Controls do not adequately reflect test conditions	Review procedure; determine whether same or different cells, reagents, or methods were used for test and control slides
	Reading error	More reading experience needed, since controls may be easier to read and interpret than test slides
	Reagent cross-contamination	Take care to confine reagents to wells
	Inoculum debris or microorganisms on the test slide	Increase number of washes performed on monolayers prior to harvesting

[a]Reprinted from Isenberg (1998) with permission of the publisher.

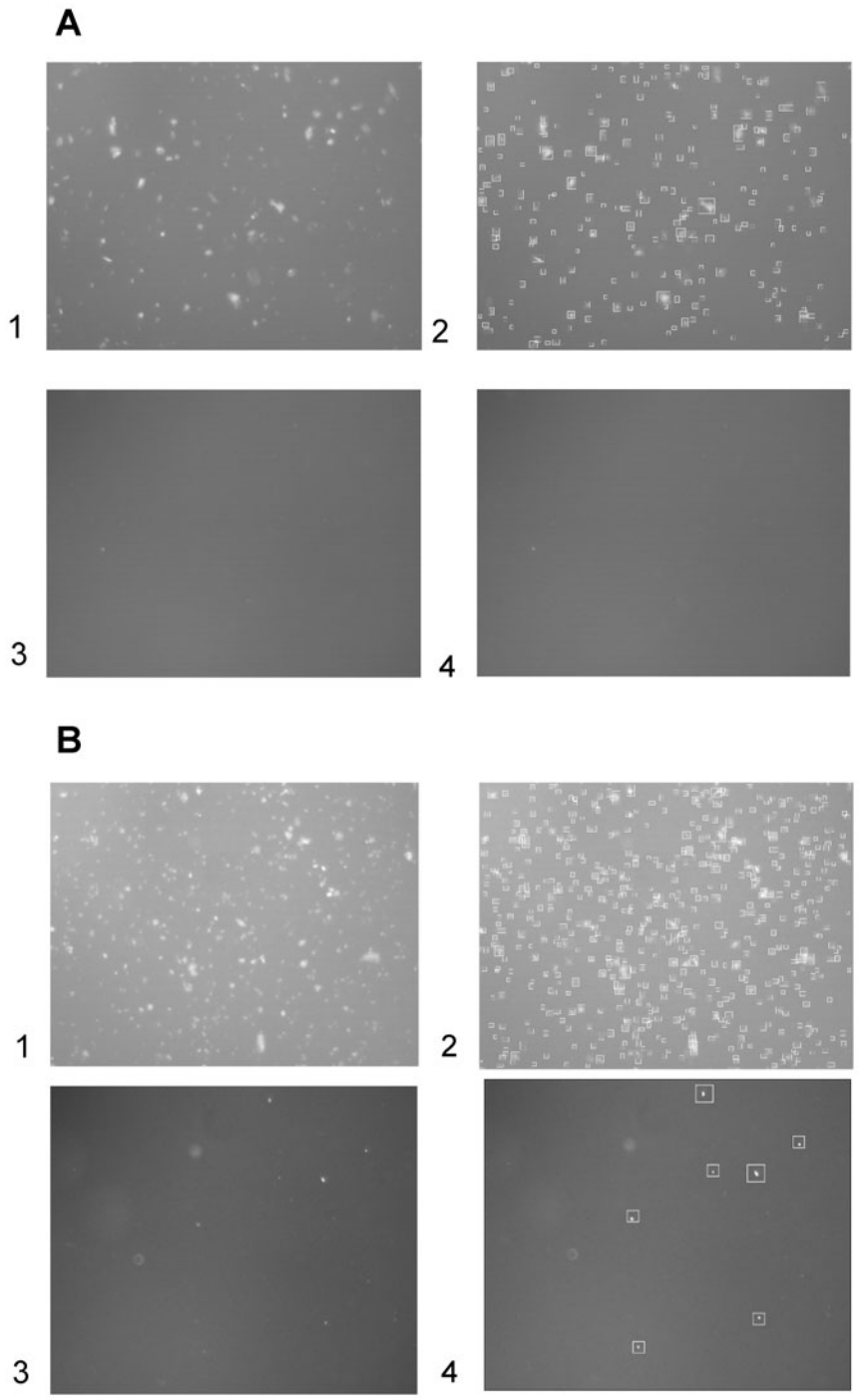

FIGURE 4 Analysis of respiratory specimens known to be negative (A) and positive (B) for influenza A virus using FITC-labeled influenza-specific MAb reagents. Images 1 and 3: primary image capture using Evans blue and FITC filters, respectively; images 2 and 4: software algorithm analysis of images 1 and 3. The boxed cells in images 2 and 4 were analyzed for colocalized fluorescence signal. Seven influenza-positive cells were identified (panel B, image 4), thus indicating a "positive" result.

Care must be taken with passing and maintaining clinical isolates used as controls, particularly RNA viruses. Passing an RNA virus at a high multiplicity of infection may cause an increase in the production of defective interfering particles (hence a lower infectivity of the virus stock) and reading errors during RNA replication, resulting in mutations in the RNA and leading to poor or absent staining due to the loss of target epitopes. Similarly, at high passage numbers, some cell lines are no longer susceptible to infection by certain viruses, e.g., HSV infection of A549 cells.

RECENT AND FUTURE ADVANCES

The past several years have seen few changes in the field of fluorescence-based antibody assays. Some of these have been described earlier in this chapter, such as the use of mixed-cell cultures in shell vial assays or the replacement of rhodamine with R-phycoerythrin in dual fluorescence assays. One major advance has been the introduction of automation. Instruments are now commercially available that were initially designed to automate immunofluorescence assays for the diagnosis of autoimmune disorders: the BioRad, Inc. (Hercules, CA) PhD system, the AP22 Speedy IF and AP16 IF Plus, manufactured by DAS (Rome, Italy), and the AFT2000 by Immuno Concepts (Sacramento, CA). Each instrument has the ability to pipette DFA reagents into the wells of multiwell microscope slides, followed by wash steps after an appropriate incubation period. Preparation (deposition of the patient specimen on the slide), and acetone fixation, still need to be performed manually.

Development of sophisticated imaging algorithms capable of analyzing a blood specimen for the presence of cancerous cells has recently been commercially introduced (Cell Tracks II) (Larson et al., 2004). Adaptation of this technology to infectious diseases has significant potential to make the use of fluorescent immunoassays more widespread, and applicable to the rapid, sensitive, and specific detection of a broad array of pathogenic agents, most notably viruses, bacteria, and parasites.

The general concept effectively uses all of the principles of fluorescence described earlier but would eliminate the requirement for subjective interpretation through the use of instrumentation. Such a prototype instrument has been developed that combines the following four elements for reliable, fast, objective interpretation: (i) excitation of fluorophores in a cellular specimen using two (or more) "super bright" light-emitting diodes (LEDs); (ii) optical filtration, or separation of emitted signals using dual (or more) wavelengths within the fluorescent spectrum required for detection of common cellular and antibody stains (e.g., Evans blue [550 nm] and FITC [520 nm], respectively); (iii) a single objective lens coupled to a charge-coupled device camera for image capture; and (iv) "smart, objective analysis" by a proven software algorithm that is "trained" to differentiate interrogated images based on pattern recognition properties of color, shape, size, and signal intensity.

By the addition of a motorized stage for processing glass slides which contain the clinical specimen and complementary user interface software, the technology comprises an automated system potentially able to provide highly sensitive and specific analytical results, eliminating subjective visual interpretation by a laboratory technologist. Moreover, to ensure specimen quality, automated enumeration of the number and type of cells contained in the specimen could be readily determined during the interrogation by the instrument.

An example of using automated cell analysis for directly identifying influenza A virus in cells derived from a patient specimen is demonstrated in Fig. 4. Figure 4A contains camera and algorithm "interrogated" images of epithelial cells obtained with a nasopharyngeal swab from a patient not infected with influenza A virus, and stained with a reagent containing Evans blue and FITC-labeled MAbs to influenza A. Figure 4B contains images of cells similarly obtained and stained from a patient subsequently determined by cell culture to be infected with influenza A virus. Using discriminatory parameters of size, shape, signal intensity, and the fundamental requirement that both the Evans blue and FITC signals co-localize to the identical "cell image," the algorithm is able to sensitively and specifically distinguish and identify influenza-infected cells from uninfected cells.

Widespread use of automated imaging technology for clinical virology will require the further development of specific MAb reagents for specific viruses and a rapid and easy format to enable users to process specimens quickly. The health benefits to patients and the practical diagnostic benefits to laboratory professionals and physicians derived from this technology are numerous and may enable the use of IFA in settings lacking technologists with the interpretive training and experience currently required to perform IFA analysis. This is particularly true in the case of rapid influenza detection where approved antiviral drugs are available for patient management. Assuming the processing methodology can be reduced to <10 min per specimen, automated image analysis could provide a DFA solution with significantly increased sensitivity and specificity in an application environment currently reserved for rapid yet traditionally less sensitive point of care devices.

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Enzyme Immunoassays and Immunochromatography

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7

Laboratorians continually seek methodologies that yield accurate results in a timely fashion, are cost effective, and require less technical expertise. Enzyme immunoassay (EIA) and immunochromatography (lateral flow) (ICR) are two technologies that have been applied successfully in viral diagnostics for identification of viruses isolated in cell culture, detection of viral antigens in clinical samples, and identification of viral antibodies in serum. EIA is used here to describe a variety of assays that are based on reactivity of antibodies with their antigens and detection of this reactivity through use of components conjugated with active enzymes; these enzymes subsequently act on their substrate to produce a color change. In most of these assays, one of the components is bound to a solid phase, thus facilitating separation of antigen-antibody complexes from the solution. These are called heterogeneous EIAs.

ICR is used here to describe assays that rely on the binding of antigens with antibodies as the components diffuse along a nitrocellulose strip; visualization of the reactions is possible because one of the components is labeled with some sort of colored particle that allows captured antigen-antibody complexes to produce a macroscopically visible area of reactivity. This chapter deals with principles of EIA and ICR and contemporary applications of both methods in viral antibody and antigen detection in the diagnostic virology laboratory.

EIA

Based on the discovery that antibodies could be labeled with enzymes without losing their immunologic reactivity, EIAs were developed and have experienced broad application in the diagnostic laboratory. For more than 3 decades, scientists have seen applications of EIA in detecting viral antibodies and antigens. Many EIAs for viral antibody and antigen detection are currently U.S. Food and Drug Administration (FDA) cleared and marketed commercially as test kits that include all required reagents and provide exact performance specifications. EIA concepts and contemporary applications in diagnostic virology will be discussed here. Assay development and detailed descriptions of EIA reagents and components will not be presented here, but this information has been published previously (Crowther, 2001; Wild, 2005). Procedural steps for assay performance of specific assays also will not be included here because each commercially marketed product must be used in accordance with the manufacturer's instructions, which are unique for each product.

Many configurations and variations of EIA technology are used in diagnostic virology laboratories, and descriptions of these formats follow. EIAs, in general, are popular because most are suitable for testing of large numbers of samples, require little technical expertise to perform, and are generally cost-effective. Many of the color change reactions of the EIA systems are objectively measured by spectrophotometry, which eliminates subjective assessments by technologists and provides results in the form of continuous-scale numerical readings.

Immunoperoxidase Staining (Histochemical EIA, Immunohistologic Staining)

Immunoperoxidase staining, also called histochemical EIA or immunohistologic staining, is a type of EIA. "Immunoperoxidase staining" is the term used to describe these assays because most involve the enzyme horseradish peroxidase. Tissue sections, other clinical samples such as cells from lesions, the respiratory tract, or the genital area, or cells from virus-infected cell cultures are fixed on microscope slides. Antibodies of known specificity are then added. In direct staining, the antibodies are labeled with horseradish peroxidase enzyme. After an incubation period and rinsing, a substrate solution is added; in areas where peroxidase-labeled antibodies have bound, the peroxidase enzymes act on the substrate to produce an insoluble colored reaction product. Direct staining usually requires only 20 to 30 min. In indirect staining, the antibodies applied to the specimen are unlabeled. After incubation and rinsing, a preparation of horseradish peroxidase-labeled antispecies antibodies (directed against the species in which the primary antibody was raised) is added. These "detection" antibodies bind to unlabeled antibodies that were bound in the first step. After incubation and rinsing, a substrate solution is added and color development occurs. Indirect staining requires approximately 90 min. Results for both direct and indirect staining are evaluated by viewing the preparation through a standard light microscope. The intensity, distribution, and pattern of the staining are evaluated.

Immunoperoxidase staining has been applied in the clinical laboratory to detect many viral antigens and antibodies, although this staining is not used as frequently as

immunofluorescence. The obvious advantage of immunoperoxidase over immunofluorescence is that a fluorescence microscope is not required for evaluation of immunoperoxidase stains; a standard light microscope is all that is necessary. Disadvantages of immunoperoxidase methods include the additional time required for color development during the staining process and nonspecific staining that may be due to indigenous peroxidases in some types of clinical specimens. Immunoperoxidase detection of viral antigens in cells is described in detail in chapter 8 of this volume.

Immunoblotting

Immunoblotting is another application of EIA used in the clinical virology laboratory. Blotting, which refers to the transfer of DNA, RNA, or protein from electrophoretic gels to a membrane, is used to prepare the antigen, and an EIA method is used to react antibodies with the blotted antigen to identify or characterize either the blotted antigen or the antibodies. In immunoblotting, the antigen used in the test system is often a protein antigen that has been blotted using the Western blotting technique in which an electrophoretically separated antigen is blotted onto nitrocellulose paper and the paper is dried and cut into strips. In other immunoblotting assays, cloned antigens are applied in bands to a test strip.

Immunoblotting is performed by flooding the immunoblot antigen strip with antibodies. Then, following incubation and rinsing, the appropriate enzyme-labeled "detection" antibody is added, followed by substrate. Reactivity is signaled by the presence of colored bands at appropriate positions and of sufficient intensity on the strip. Immunoblotting is one of the most commonly used methods for evaluating antibodies produced in infections with human immunodeficiency virus (HIV) types 1 and 2, human T-cell lymphotropic virus types 1 and 2, and hepatitis C virus (HCV). Applications of immunoblotting for the diagnosis of viral infections are presented in chapter 13 of this volume.

Noncompetitive Solid-Phase EIAs

Noncompetitive solid-phase EIAs are the type used most commonly in clinical virology laboratories. These feature a solid phase to which either a known antigen or a known antibody is bound. The solid phase may be the wall of a test tube or microwell, the surface of a plastic bead, or some other platform. For detection of unknown antibodies, a known viral antigen is bound to the solid phase, and the "test" material is the patient's serum or plasma. For detection of unknown antigen, a known viral antibody is bound to the solid phase, and the test material is a clinical sample (e.g., material collected from the patient's throat, lesion, or genital area) or a suspension of virus-infected cells from a cell culture. After the test material is allowed to react with the solid phase and the unbound reactants are rinsed away, there is subsequent addition of enzyme-labeled "detection" antibodies. Detection antibodies bind either to antigen that was captured during the first incubation period (in an antigen detection EIA) or to the patient's antibodies that attached to antigen on the solid phase during the first incubation period (in an antibody detection EIA). After washing away of unattached components, a substrate solution is added. The enzymes attached to the detection antibodies act on the substrate solution, resulting in a change in color. The color change may be measured visually but is often quantitated spectrophotometrically. EIAs using the testing sequence just described are called noncompetitive EIAs. A tube-based noncompetitive EIA for identification of antibodies is diagrammed in Fig. 1. This features known viral antigen bound to the wall of the test tube and procedural steps of addition of the patient's serum, incubation and rinsing, addition of a detection preparation of enzyme-labeled anti-human immunoglobulin G (IgG), with additional

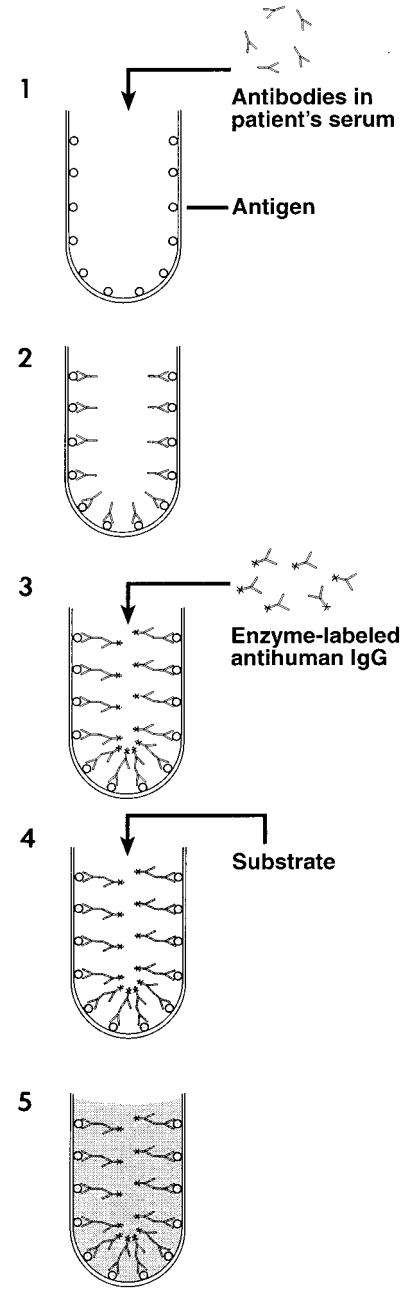

FIGURE 1 Tube-based noncompetitive EIA for antibody detection. (Step 1) The patient's serum is added to a tube or microwell coated with known antigen. (Step 2) Patient's antibodies bind to the antigen. Unbound components are washed away. (Step 3) Enzyme-labeled anti-human IgG is added. This binds to antibodies that bound in step 2. Unbound components are washed away. (Step 4) A substrate solution is added. (Step 5) The enzymes that are part of the enzyme-labeled anti-human IgG act on the substrate to produce a color change.

incubation and rinsing, followed by addition of substrate solution.

The use of a plastic bead as the solid phase in a noncompetitive antibody detection EIA system is shown in Fig. 2. Known viral antigen is bound to the surface of the bead. The antigen-coated bead is incubated in a dilution of the patient's serum and then rinsed before the addition of enzyme-labeled anti-human IgG detection antibodies; these bind to any IgG that attached to the antigen during the initial incubation period. After incubation and rinsing, the substrate solution is added. Color change will be observed if the patient's antibody bound in the initial incubation; no color change will occur if the patient's antibodies did not bind in the initial step. Color development is measured spectrophotometrically, producing a continuous scale numerical readout. Most noncompetitive EIA systems, regardless of the form of the solid phase, require approximately 45 min to 3 h to complete and can be performed manually or automated via various instrument systems.

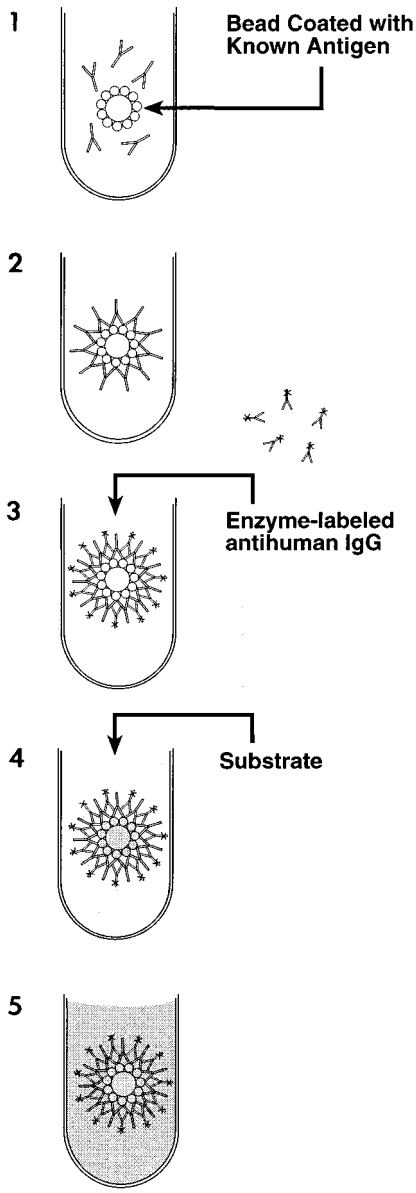

FIGURE 2 Bead-based noncompetitive EIA for antibody detection. (Step 1) An antigen-coated bead is incubated in a dilution of the patient's serum. (Step 2) Patient's antibodies bind to the antigen. Unbound components are washed away. (Step 3) Enzyme-labeled anti-human IgG is added. This binds to antibodies that bound in step 2. Unbound components are washed away. (Step 4) A substrate solution is added. (Step 5) The enzymes that are part of the enzyme-labeled anti-human IgG act on the substrate to produce a color change.

In noncompetitive antigen detection EIAs, the patient's clinical sample (a fecal sample, throat sample, lesion sample, or serum) or a suspension of virus-infected cells from a cell culture is exposed to an antibody-coated solid phase coated with antiviral antibodies (Fig. 3). The antibodies on the solid phase function in capturing any viral antigen that is present in the sample. Subsequently or concurrently, enzyme-conjugated antiviral detection antibodies are added and bind to the viral antigen that was captured by the antibodies on the solid phase. After incubation and rinsing, a substrate solution is added and a color develops. Color change is usually measured spectrophotometrically, providing a continuous-scale numerical value. As with noncompetitive EIA methods for antibody detection, the entire process takes approximately 45 min to 3 h and can be completed manually or by automated systems.

Competitive Solid-Phase EIA

In competitive solid-phase EIA systems, as in noncompetitive solid-phase systems, either known antigen or a known antibody is attached to the solid phase, and this solid phase is exposed to the patient's sample. In contrast, in the competitive system, a measured amount of known enzyme-labeled component (of the same specificity as the component being detected in the assay) is added along with the patient's sample (Fig. 4). Hence, the labeled component competes against the unlabeled component in the patient's sample for binding sites on the solid phase. After incubation and rinsing, which take approximately 1 h, the solid phase is exposed to a substrate solution. If the patient's sample contained a large amount of the component in question, the patient's unlabeled component would have occupied most or all of the available binding sites, and little, if any, of the enzyme-labeled component (added in the EIA system) would bind to the solid phase; therefore, slight or no color change would result. If the patient's sample contained little or none of the component in question, binding sites on the solid phase would be available, and the enzyme-labeled component (added in the EIA system) would bind, resulting in color change when the substrate solution was added. Thus, in the competitive EIAs, an absence of color change signals the presence or positivity of the desired analyte while color change indicates absence or negativity.

Chemiluminescence and Biotin-Avidin EIAs

EIAs are sometimes named by their detection system. Chemiluminescence and biotin-avidin EIAs are two of these. Traditional EIA-type steps involving solid-phase reactants and enzyme-labeled detection complexes are performed. In chemiluminescence, the enzyme label may be horseradish peroxidase. The enzyme acts on a substrate solution containing pyrogallol, isoluminol, acridinium ester, or oxalate ester, oxidizing these substrates to produce a burst of light (luminescence). This burst can be measured by a luminometer. The amount of luminescence, like the color change

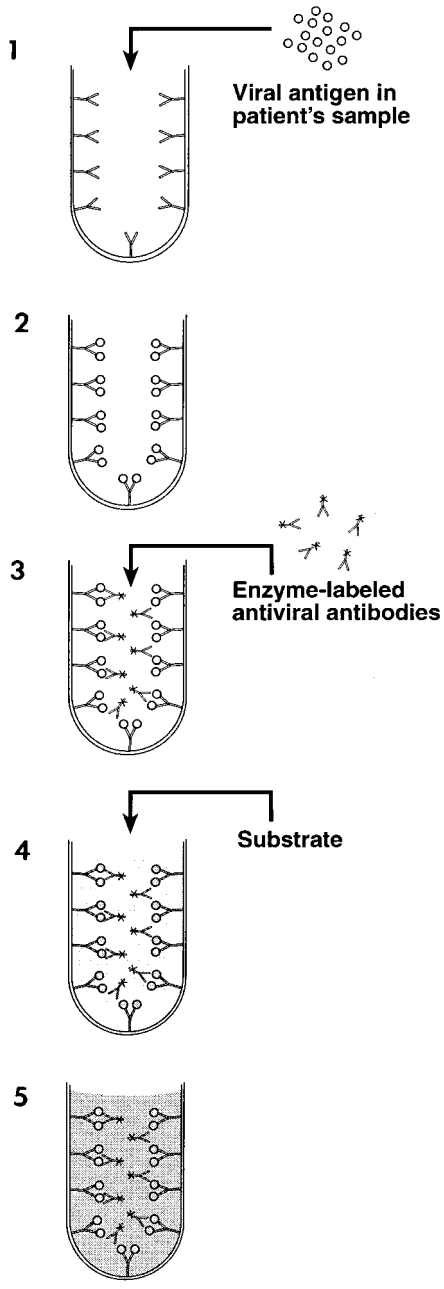

FIGURE 3 Noncompetitive tube-based EIA for antigen detection. (Step 1) The patient's sample (feces, throat or nasal wash, etc.) is added to a tube or microwell coated with antiviral antibodies of known specificity. (Step 2) The antibodies coating the tube recognize and bind (or capture) the antigen in the patient's sample. Unbound components are washed away. (Step 3) Enzyme-labeled antiviral antibodies are added. They bind to the viral antigens captured in step 2. Unbound components are washed away. (Step 4) A substrate solution is added. (Step 5) The enzymes that are part of the bound enzyme-labeled antiviral antibodies act on the substrate to produce a color change.

in traditional solid-phase EIAs, is proportional to the amount of antigen-antibody binding.

In biotin-avidin EIAs, biotin-conjugated primary antibodies are incubated with the test sample. Unbound

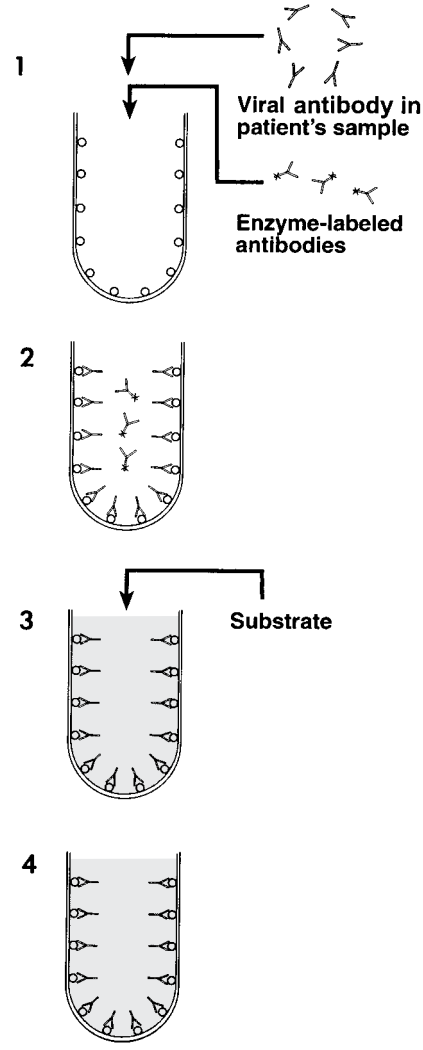

FIGURE 4 Competitive tube-based EIA for antibody detection. (Step 1) The patient's serum and enzyme-labeled antibodies are added to an antigen-coated tube or microwell. (Step 2) The patient's antibodies compete with the enzyme-labeled antibodies for binding to the antigen. If a sufficient quantity of the patient's antibodies are present, they bind to the antigen, and few, if any, of the enzyme-labeled antibodies succeed in binding. Unbound components are washed away. (Step 3) A substrate solution is added. (Step 4) There is no color change because unlabeled antibodies from the patient's serum have bound and few, if any, enzyme-labeled antibodies have bound.

antibodies are washed away. Then enzyme-conjugated avidin or streptavidin is added; one molecule of avidin can react with four biotin molecules and bind strongly in a reaction that is nonimmunological and nearly irreversible. After addition of a substrate solution, a color change is observed. This system avoids the nonspecificity of other EIAs involving antispecies antibody (Forghani and Hagens, 1995).

Automation of Competitive and Noncompetitive EIAs

To handle testing of large numbers of samples in an efficient, cost-effective, and labor-saving manner, many of the

microwell-based EIAs, both competitive and noncompetitive, are now automated. The basic principle of these procedures is the same as for the comparable manual EIAs, except that, in the automated systems, an instrument dilutes, transfers, mixes, rinses, and evaluates results for all of the assays.

For microwell-based EIAs, the technologist "builds" a microplate by assembling microwells coated with the antigen that will be used in the test system. This microwell plate is positioned on the instrument along with the necessary reagents (diluent, conjugate, substrate) and tubes containing undiluted patients' serum samples that will be assayed for the presence of antibodies. After the technologist positions all reagents and samples as required, the instrument is activated, and its microcomputer directs the instrument through the appropriate timed cycles of dilution, reagent addition, incubation, washing, and spectrophotometric evaluation of reactions. With these instruments, a single immunoassay (e.g., rubella IgG) can be performed on all of the samples, or two or more compatible assays (eg.. rubella IgG and cytomegalovirus IgG) can be performed simultaneously on all of the samples. Likewise, multiple (often 3 to 8) compatible separate assays may be performed on limited numbers of samples in the same run of testing.

One automated system for EIA testing, the bioMèrieux VIDAS (bioMèrièux, Inc., Durham, NC), features an unusual configuration. The solid-phase in this system looks like a typical disposable pipette tip from a piston-type pipetting device. The inside surface of this tip, which is called a solid-phase receptacle (SPR), is coated with either known antigen or known antibody. The steps in the EIA procedure are all performed by the instrument and involve drawing up various fluids (i.e., patient's sample, wash buffers, conjugated antibody solutions, and substrates) in sequence into the SPR, where the reactions occur. The VIDAS reagent chamber is also unique, featuring a strip of sealed compartments. As the EIA process proceeds, the SPR pokes through the covering on the sealed compartments to access the reagents and draw them up into the SPR. The VIDAS instrument completes all incubation, washing, and reading steps automatically.

For other automated EIAs, the test containers (microwells or some other test vessel configuration) are stored onboard the instrument, along with all reagents. The only work of the technologist is simply to enter manually or scan via a bar code reader the log-in numbers and the names of the tests to be performed on each sample and position the sample in the instrument. In larger laboratories, even these functions may be accomplished by robotic systems that read bar coded information, program instrumentation, and place test samples into position in the instrument. The number of samples that can be tested is enormous, with high throughput.

Automation is effective, regardless of whether the EIA is designed to detect unknown antigens or antibodies and regardless of the readout (color change versus chemiluminescence). This is popular and essential in many diagnostic laboratories, facilitating testing of large volumes of specimens, eliminating human error in pipetting and washing steps, and providing electronic transmission of results from the instrument directly into the laboratory's information system. In addition, instrumentation provides for simultaneous performance of several assays at the same time and requires less expertise on the part of the operator. Once the instrument is programmed and supplied with all of the necessary samples and reagents, it completes all phases of testing and holds the data for the technologist to harvest. The more-sophisticated instruments are capable of performing a wider variety of assays. All of these advances are helping to lower laboratory costs while providing high-quality assay results with a shorter turnaround time.

Membrane EIAs

Rapid (20 to 30 min) EIA systems are available in which the test system is a single-use, self-contained unit assembled in individual modules or cassettes. These systems have been applied for detecting both unknown antibodies and unknown antigens. The most prominent application of membrane EIAs in diagnostic virology at this writing is detection of viral antigens, most commonly either influenza A and B or respiratory syncytial virus (RSV), in patients' samples collected from the respiratory tract. One EIA system, the Directigen EIA (Becton Dickinson, Sparks, MD), includes a pretreatment step, after which the sample is forced through a focusing device, resulting in nonspecific adherence of viral antigen on the membrane held within the test cassette (Fig. 5). Then sequential applications of enzyme-labeled antiviral antibodies, washing buffer, and substrate solution are applied to the cassette. Color development occurs on the pad in the packet if viral antigen was present in the sample. This pad is often prepared in a unique shape that facilitates interpreting the color change reaction. The EIA test area also contains an internal control to monitor the performance of both the assay and the user. The internal control is actually a dot of viral antigen. There should be color development of this control if the reagents are working properly and the test is performed correctly, regardless of whether the patient's sample is positive or negative for the analyte. These assays have several timed steps as part of the procedure. Although necessary technical expertise is minimal, most of these assays are classified as moderately complex according to the Clinical Laboratory Improvement Act (CLIA).

Optical Immunoassays

Another variation on the principle of EIA is marketed in a format called optical immunoassay (OIA; Thermo Electron Corp., formerly Thermo Biostar, Inc., Boulder, CO). In OIA systems, the surface of a silicon wafer with an optical coating serves as the solid-phase support matrix (Fig. 6). Like membrane-based EIAs, OIA systems are produced in self-contained single-use units. In OIA systems for antigen detection, a capture antibody is attached to the solid-phase surface. White light reflected through this surface appears golden in color unless the thickness of the molecular thin film is changed. When a clinical sample containing the desired antigen is placed on the test device, the capture antibody binds the antigen. In steps that are typical of an EIA, enzyme-conjugated antibodies are added that bind to the captured antigen. A substrate solution is then added. The binding of components, along with precipitation of the colored substrate, increases the thickness of the molecular film, providing a "dual readout" involving changes in the optical quality of reflected light and a color change of the substrate. In a positive result, a purple color can be seen covering most of the test area. The silicon wafer contains an internal control composed of inactivated virus to monitor the performance of both the assay and the user. If the assay is performed correctly and the reagents are working as they should, this dot will change color—regardless of whether the patient's sample is positive or negative for the analyte in question. This testing takes only 15 to 20 minutes to complete and requires no special expertise or laboratory equipment. Although OIA has a unique appearance, it is basically

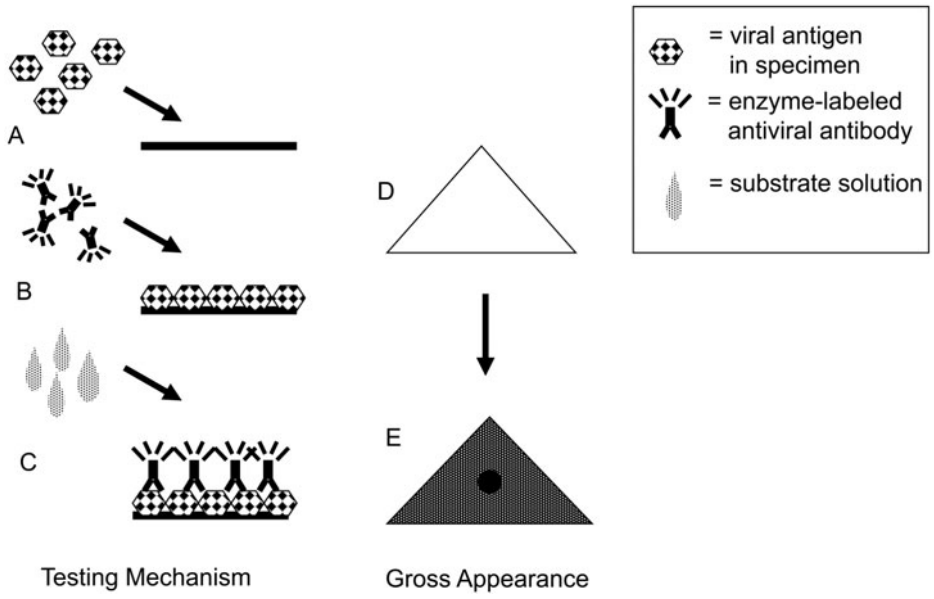

FIGURE 5 Membrane EIA testing mechanism and gross appearance. Testing mechanism: (A) viral antigen in specimen is nonspecifically adhered to the membrane by filtration through a focusing device; (B) enzyme-labeled antiviral antibody is added and binds to viral antigen present on the membrane; (C) a substrate solution is added and changes color when acted upon by the enzyme. Gross appearance: (D) a test area is defined within the cassette—this is colorless at the beginning of the assay; (E) the test area changes color due to the action of the enzyme on the substrate solution. The colored dot in the center of the test area is a built-in control to ensure that the device is functioning properly.

another variation of EIA. Like the membrane-based rapid EIAs, OIAs have several steps involved in testing and are classified as moderately complex according to CLIA.

ICR

ICR, also referred to as "lateral flow," relies on migration via capillary action of antibody-antigen complexes over a nitrocellulose strip. The point of origin where the test sample is placed is separate from the area of the strip where the assay result is read. The technique can be used for detection of either unknown antigen or unknown antibodies. Figure 7 shows the principles of an antigen-detection ICR. In this testing, antiviral monoclonal antibodies are labeled with visualizing particles. These particles are often colloidal gold nanoparticles, carbon black, or blue polystyrene. The labeled antibodies, sometimes called the "signal" or "detection" antibodies, are adsorbed nonspecifically onto one end of the strip. This end of the strip is where the patient's sample is applied (Fig. 7A). At the opposite end of the test strip, there is an area, the test area, in which unlabeled antiviral antibodies, usually polyclonal, are immobilized in a thin line. Further toward the opposite end of the strip, there is an area, the control area, in which unlabeled polyclonal anti-species (usually anti-mouse) IgG is immobilized.

In ICR antigen assays the patient's sample may be one that has been collected on a swab and diluted in a small amount of buffer or extracting compound or may be a drop of liquid sample from various aspirates or washes, etc. If the sample contains the antigen in question, the labeled monoclonal antibodies on the sample pad will bind to the viral antigen, and the labeled antiviral antibody-viral antigen complexes will travel laterally along the test strip membrane (Fig. 7B, C). When they reach the test area in which the unlabeled antiviral antibodies are bound, these antibodies will bind to the viral antigens—the ones that were initially bound to the labeled monoclonal antibodies, resulting in a visible colored line (usually pink or red in color). Unbound or excess labeled antiviral antibodies pass through the test line and are bound at a control line by the anti-mouse IgG, also resulting in a visible colored line (Fig. 7D). The presence of a line at the test area and at the control area indicates a positive result. The appearance of a line at the control area in the absence of a line in the test area indicates a negative result. The assay result cannot be interpreted unless a line is seen in the control area.

If an ICR assay is designed for detecting unknown antibodies, the known component in the test system is antigen. The actual mechanisms of these assays vary, and most are proprietary. In one antibody detection ICR, patient's antibodies (in blood, serum, or plasma) migrate through an area on the test strip impregnated with recombinant protein antigens linked to colloidal gold. Labeled antigen-antibody complexes form, continue to migrate, and then bind in the test area where unlabeled recombinant proteins are immobilized; a visible pink/red bind results (Uni-Gold Recombigen HIV; Trinity Biotech, Wicklow, Ireland, and Jamestown, NY). In another antibody detection ICR, the patient's antibodies travel along the test strip through a zone that is impregnated with a proprietary colorometric reagent composed of protein A and colloidal gold. At the test line, viral antigens are immobilized on the strip. If the patient's sample contains antibodies that react with the antigen, a reddish-purple line will appear at the test line (OraQuick Advance Rapid HIV-1/2; OraSure Technologies, Inc., Bethlehem, PA). Both

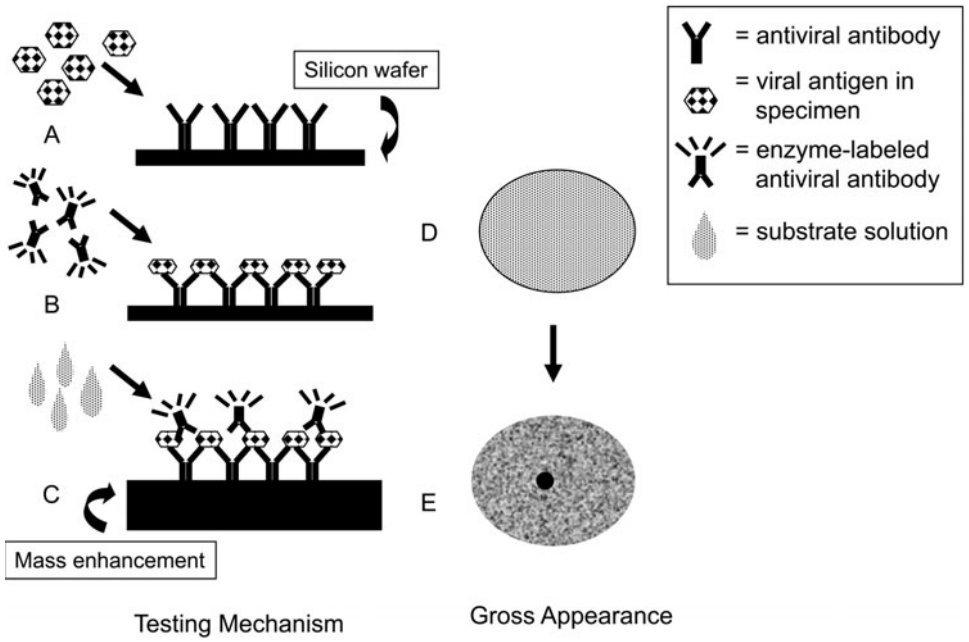

FIGURE 6 OIA testing mechanism and gross appearance. Testing mechanism: (A) viral antigen in the specimen is added to a silicon wafer coated with antiviral antibody; (B) enzyme-labeled antiviral antibody is added and binds to viral antigen bound in the previous step; (C) a substrate solution is added and changes color when acted upon by the enzyme—this is enhanced by mass enhancement due to the change in thickness of the silicon wafer produced by antigen/antibody complexes and precipitated substrate, thus changing the reflectance of light off the wafer. Gross appearance: (D) the surface of the silicon wafer is mirror-like; (E) a change in the color of the test area and of light reflected from the wafer is seen due to color change and mass enhancement. The colored dot in the center of the test area is a built-in control to ensure that the device is functioning.

of these ICR systems for detection of unknown antibodies include a control area to indicate that the test device is functioning correctly.

Most ICR assays are one-step procedures. Following addition of the patient's sample, the test requires no further manipulation other than observation of the result at the end of the time period. Many of the ICR assays have been granted waived status according to CLIA guidelines. This facilitates performance of these assays in physicians' offices and clinics. They are user-friendly, require 15 to 30 min to complete, are stable in the long term, and cost $18 to $22 per test.

EIA, OIA, AND ICR APPLICATIONS IN DIAGNOSTIC VIROLOGY

Detecting Viral Antigens

EIA, OIA, and ICR for viral antigen detection, in contrast to the immunofluorescence assays for viral antigen detection and virus isolation procedures, have less stringent requirements for specimens. For immunofluorescence work, samples must contain intact infected cells. EIA, OIA, and ICR methods can detect free virus, so intact infected cells are not essential for accurate results. For virus isolation, viable infectious virus must be present in the sample. EIA, OIA, and ICR methods successfully detect noninfectious viruses and viral antigen fragments, which would fail to proliferate in cell cultures. However, falsely negative viral antigen ICR results have been reported with bloody samples and those with high viscosity (Kuroiwa et al., 2004). The type of specimen can be important, and manufacturer's information concerning specimen type and handling must be followed.

Either one or two types of viral antigen can be detected with each of the rapid EIA, OIA, or ICR viral antigen assays; therefore, if viruses other than the target virus are present in the sample, they will remain undetected. Virus isolation remains the most thorough method of detecting all viruses present in a sample.

Detection of antigens of some viruses (e.g., hepatitis B, rotavirus) represents the only practical approach for diagnosis because these agents do not proliferate in standard cell cultures. Detection of antigens of some other viruses, especially the common respiratory viruses (i.e., adenovirus, influenza A and B viruses, parainfluenza viruses 1, 2, and 3, and RSV), has been shown to be more useful in patient management than either traditional virus isolation (Woo et al., 1997; Barenfanger et al., 2000; Bonner et al., 2003) or viral detection in rapid culture using centrifugation-enhanced inoculation (Adcock et al., 1997). Children with RSV (Adcock et al., 1997) or influenza A or B virus (Bonner et al., 2003; Benito-Fernandez et al., 2006) antigen confirmed by EIA received antibiotic therapy for fewer days than antigen-negative children, and physicians felt that the rapid EIA results influenced their antibiotic decisions. Rapid diagnosis of respiratory viral infections significantly reduced hospital stays, antibiotic use, and laboratory utilization, resulting in cost savings and improved patient management (Woo et al., 1997; Barenfanger et al., 2000).

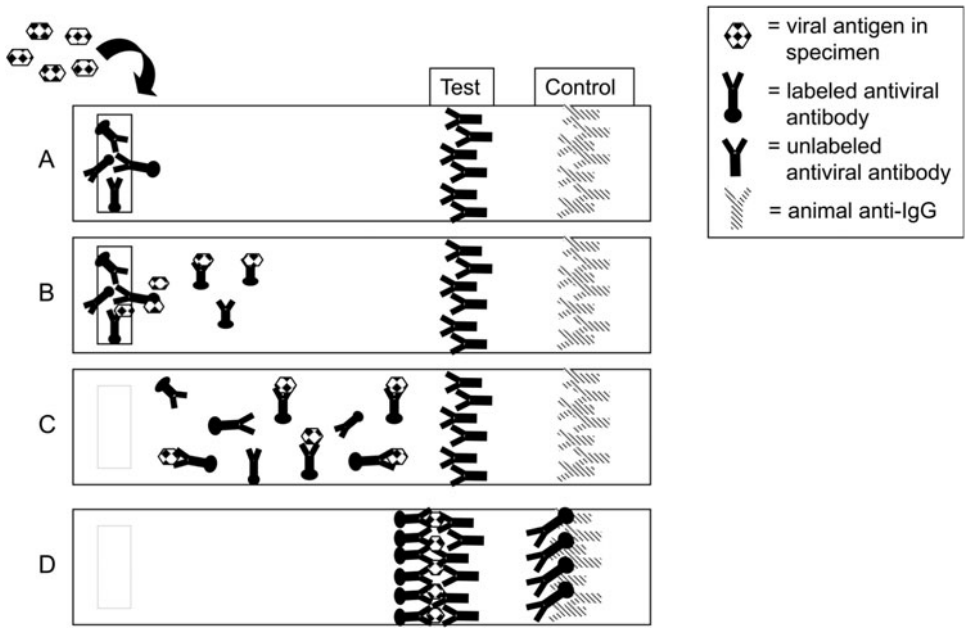

FIGURE 7 ICR (lateral flow) testing mechanism. (A) Viral antigen in the specimen is added to the sample port of a nitrocellulose strip; nonspecifically bound labeled antiviral antibodies are present in the specimen area. The nitrocellulose strip also includes a test area of unlabeled antiviral antibodies and a control area of unlabeled animal antihuman IgG. (B) Viral antigen in the sample binds to labeled antiviral antibodies in the test area, and the complexes begin to migrate along the strip. (C) Migration continues. (D) Viral antigen, in complex with labeled antiviral antibodies, is recognized by the unlabeled antiviral antibodies in the test area of the strip and captured to form a visible line; excess labeled antiviral antibodies continue to migrate and are captured at the control line by anti-IgG. This control ensures that the specimen migrated the entire length of the strip and that the strip is functioning properly.

Of the common respiratory viruses, only RSV and influenza A and B virus antigens are frequently detected by EIA, OIA, and ICRs. Although RSV and influenza A and B viruses proliferate in standard cell cultures, with RSV producing dramatic cytopathogenic effect and influenza A and B viruses replicating more slowly and producing a more subtle cytopathogenic effect, and antigens of all three of these viruses can be identified by immunofluorescence, the rapid EIA, OIA, and ICRs are popular because they can be performed quickly and require little time or technical expertise. Studies comparing these rapid antigen assays to virus isolation in cell culture show that the rapid methods have very good specificity but moderate to poor sensitivity (Table 1). At least 100,000 viral particles must be present for the OIA rapid antigen systems to yield a positive result, and other EIA and ICR systems may require as many as 1,000,000 viral particles; this is in contrast to viral culture, which may require as few as 10 infectious virus particles for successful virus isolation (St. George et al., 2002). For EIA, OIA, and ICR, the sensitivity of RSV antigen detection compared to cell culture is higher than that of similar tests that detect influenza virus antigens (Table 1). In general, the specificities (compared to virus isolation in cell culture) reported for rapid assays for RSV and influenza virus antigen detection tend to be high (Table 1), and the predictive value of a positive result is high, especially during respiratory virus season (World Health Organization, 2005). Some report a lack of sensitivity and specificity for influenza B virus detection (Landry et al., 2000; Chan et al., 2002; Cazacu et al., 2003) in these antigen detection systems, while others (Cazacu et al., 2004a, 2004b) found that the sensitivity of influenza B virus detection was equivalent to that of influenza A virus detection.

However, there is considerable variation in findings from study to study—with differences relating to the level and type of virus circulating in the particular season, age of patients tested, skill of testing personnel, and format of the reference cell culture method (rapid culture versus conventional tube culture). The sensitivity and specificity obtained with the rapid antigen tests when used in the routine diagnostic virology laboratory are often lower than that stated by the manufacturer and lower than that reported in studies that were conducted under tightly controlled circumstances targeting a particular group of patients (Newton et al., 2002).

The EIA, OIA, and ICRs for RSV and influenza A and B virus antigens are especially useful when a rapid result is needed and trained virologists are not available. However, although the need for technical expertise is advertised as being minimal for performance of these rapid tests, testing that is carried out by technicians or other personnel who are less experienced with test kits, especially in reading results that are weakly positive, yields lower sensitivity and specificity relative to cell culture; this finding is unexpected in view of the touted simplicity of some assays (Noyola et al., 2000). There are a number of concerns for FDA-cleared CLIA-waived testing that is performed outside the laboratory at point-of-care or near-patient waived sites in physicians' offices and clinics. Mackie et al. (2004) found that 16 of 27 discordant observations reported by

TABLE 1 Rapid EIA, OIA, and ICR methods for RSV or influenza A and B antigen detection[a]

Manufacturer (location)/ test names/principle	Influenza A and B					RSV				
	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference
Becton Dickinson (Sparks, MD)/Directigen Flu A and B and Directigen RSV/EIA	72	98	89	95	Cazacu et al., 2003	77	96	88	92	Ohm-Smith et al., 2004
	93	95	83	98	Chan et al., 2002	86	93	95	91	Ribes et al., 2002
	64	99	94	93	Drinka, 2006	97	100	90	77	Slinger et al., 2004
	55	100	100	92	Dunn et al., 2003	87	89	84	90	Zheng et al., 2004
	56	100	100	54	Landry et al., 2004					
	86	94	89	92	Ruest et al., 2003					
	56	98	93	85	Weinberg and Walker, 2005					
Becton Dickinson (Sparks, MD)/EZ Flu A/B and RSV/ ICR						70	100	100	49	Kuroiwa et al., 2004[b]
						59	98	93	88	Ohm-Smith et al., 2004
						87	92	89	91	Zheng et al., 2004
Binax (Portland, ME)/Binax NOW Flu A and B and NOW RSV/ICR	70	99	86	97	Booth et al., 2006	89	100	100	95	Aldous et al., 2004
	62	96	NA	NA	Cruz et al., 2006	74	100	100	90	Borek et al., 2006
	65	98	89	93	Fader, 2005	79	100	100	95	Jonathan, 2006
	53	93	94	52	Landry et al., 2004	87	94	89	92	Mackie et al., 2004
	76	94	93	81	Weinberg and Walker, 2005	89	100	100	95	Ohm-Smith et al., 2004
						95	89	85	96	Zheng et al., 2004
Biostar (Thermo Electron) (Boulder, CO)/BioStar FluAB and RSV/OIA	54	74	73	56	Boivin et al., 2001	88	99.6	99	95	Aldous et al., 2005
	48	97	NA	NA	Hindiyeh et al., 2000					
	93	82	84	92	Rodriguez et al., 2002					
	64	95	NA	NA	Schultze et al., 2001					
Meridian (Cincinnati, OH)/ ImmunoCard Stat! Flu A and B Plus/ICR	71	99	84	97	Booth et al., 2006					
Remel (Lenexa, KS)/Xpect Flu A&B and RSV/ICR	94	100	100	98	Cazacu et al., 2004b	75	98	95	90	Borek et al., 2006

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TABLE 1 Rapid EIA, OIA, and ICR methods for RSV or influenza A and B antigen detection[a] *(Continued)*

Manufacturer (location)/ test names/principle	Influenza A and B					RSV				
	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Reference
Quidel (San Diego, CA)/ QuickVue Flu A & B/ICR	85	98	98	87	Agoritsas et al., 2006[c]					
	70	98	85	95	Cazacu et al., 2003					
	77	98	74	98	Poehling et al., 2002					
	95	76	81	95	Rodriguez et al., 2002					
	91	86	78	95	Ruest et al., 2003					
SAS (formerly QuickLab) Flu A and B/ICR						84	91	97	62	Kuroiwa et al., 2004[b]
						93	81	96	100	Slinger et al., 2004
Zyme Tx (Oklahoma City, OK)/ ZstatFLu/ICR	65	83	NA	NA	Hulson et al., 2001					
	70	92	76	90	Noyola et al., 2000					
	72	83	80	75	Rodriguez et al., 2002					

[a]Abbreviations: NA, not available; PPV, positive predictive value; NPV, negative predictive value. (Sensitivity, specificity, PPV, and NPV are all compared to virus isolation, unless otherwise noted.)
[b]Versus RT-PCR only.
[c]Versus RT-PCR and virus isolation.

point-of-care staff performing an ICR method, the Binax NOW (Binax, Portland, ME), for influenza A and B virus were reversed when testing was repeated on the same sample by laboratory staff. The authors concluded that a potential disadvantage of the simple tests is that less emphasis is placed on training and fewer restrictions are placed on the number of health care workers allowed to carry out testing. It is likely that test sensitivity and specificity suffer if those who perform waived testing are not properly trained and provided with adequate oversight. These issues and others involved with waived testing have been reviewed (Centers for Disease Control and Prevention, 2005) and should be considered if results of waived status viral antigen tests performed outside the laboratory are to be used in patient management.

Because the specificity of these systems is usually high during respiratory virus season when virus has been documented in the community by virus isolation in cell culture, positive results should be considered true positives; however, negative results should be confirmed by immunofluorescence testing, culture, or another secondary test (Hindiyeh et al., 2000; Aldous et al., 2004). Investigators also caution users that the rapid EIA, OIA, and ICR tests, when used for screening purposes in large populations, may miss infected patients and are not the most reliable laboratory tests for influenza detection (Cazacu et al., 2004a). In addition, during off season, i.e., when influenza and RSV are not documented to be prevalent in the population, the predictive value of a positive result decreases.

Because FDA-cleared membrane EIA, OIA, and ICR respiratory viral antigen detection methods are available in the United States for the detection of only RSV and influenza A and B viruses, other viruses will be missed unless additional testing methods are used. In addition, a positive rapid EIA, OIA, or ICR test does not eliminate the possibility that patients may be coinfected with another virus that may be contributing to their symptoms (Cazacu et al., 2004b). This is of particular significance when testing persons with impaired immune function and children with severe respiratory illness (Boivin et al., 2005; Foulonge et al., 2006). Therefore, if dual infections are of interest, additional testing must be done. The rapid EIA, OIA, and ICR methods may be useful in screening but should not be used without backup with cell culture for negative samples and as an adjunct for testing samples from patients with potentially mixed infections (Hindiyeh et al., 2000; Aldous et al., 2004; Cazacu et al., 2004a; World Health Organization, 2005).

An ICR for detection of human respiratory adenovirus in nasopharyngeal samples has been investigated (Fujimoto et al., 2004). The ICR showed 95% sensitivity compared to adenovirus isolation in cell culture and 92% sensitivity compared to PCR. Specificity was 100% for the ICR compared to both virus isolation and PCR. As of the time of this writing, there are no FDA-cleared respiratory adenovirus antigen detection ICRs available in the United States.

EIA and ICR methods for detection of rotavirus antigen in fecal and other types of samples are especially popular because this virus will not proliferate in standard cell cultures, and immunofluorescence techniques are not useful in detecting rotavirus antigen. The EIAs and ICRs have been shown to produce results which are, in many cases, comparable to those of electron microscopy and to latex agglutination. Similarly, antigens of the nonculturable enteric adenoviruses 40/41 in fecal samples are routinely detected by EIA. A summary of currently available rapid detection methods for rotavirus and enteric adenovirus antigens has been published previously (Farkas and Jiang, 2007). There is

one automated EIA system, the bioMérieux VIDAS (bioMérieux, Inc.), that offers rotavirus and enteric adenovirus antigen detection assays.

Another virus whose presence is usually confirmed through antigen detection is the hepatitis B virus (HBV). HBV antigens are found at high titers in peripheral blood, which is the specimen of choice for HBV antigen testing. HBV does not proliferate in standard cell cultures and cannot be detected through immunofluorescence, so EIA is a logical choice for antigen detection. Although the original antigen detection methods for HBV were radioimmunoassays, most have been converted to EIAs. These EIAs are most often the microwell-based assays that can be automated. EIAs for HBV antigen detection are usually performed in conjunction with EIA testing for a variety of markers of HBV infection; this represents some of the highest volume viral marker testing offered in clinical laboratories. Within the past 10 years, many of the hepatitis A virus, HBV, and HCV EIAs have been successfully automated. These high-volume assays are effectively handled by automated EIA systems.

The p24 antigen of HIV can be detected in peripheral blood samples by an EIA. Because HIV does not proliferate in standard cell cultures and is not detectable directly through immunofluorescence, EIA is a logical approach, and many antibody markers of HIV are tested by EIA. In the 1980s and 1990s, the HIV p24 antigen EIA was an important tool in early diagnosis of HIV type 1 (HIV-1) infection, in monitoring treatment of infected individuals, and in screening donated blood in the United States. However, this assay has largely been replaced by more sensitive nucleic acid tests. At this writing, the p24 EIA is commercially marketed in the United States for research use only. However, the p24 EIA is still used in other parts of the world in which access to molecular testing may be limited and the lower cost and ease of performance of the p24 EIA make it the assay of choice (Griffith et al., 2007).

Detecting Viral Antibodies

The assortment of commercially available EIA diagnostic test kits designed for detection of various viral antibodies is enormous. These require little technical expertise and provide timely, cost-effective results. EIAs have replaced many of the older, more labor intensive antibody detection methods. The decision to select one EIA over the other may be difficult, with the decision being made based on availability of personnel, testing volume, and expertise required. Most commercially available viral antibody detection kits and systems are distributed along with specific testing protocols. These protocols should be followed without modification to ensure accurate test results. No EIA testing procedures are presented in this text.

Manual bead-based EIAs are widely available for measurement of antibodies and antigens associated with the blood-borne pathogens HBV, HCV, HIV, and human T-cell lymphotropic virus. Each of the bead-based EIAs follows similar EIA steps and requires 1 to 3 h to complete. Instrumentation is available to aid with some steps of the bead-based EIAs, but total automation has not been achieved. Bead-based EIAs are also available for hepatitis A virus antibodies. Assays for analytes measured in many of the bead-based assays have been converted to other EIA formats suitable for large-scale automation.

Manual EIA systems with testing performed in microwell plates are available for detecting antibodies against cytomegalovirus, herpes simplex virus, HIV, Epstein-Barr virus, measles, mumps, rubella, varicella-zoster viruses, and perhaps, others. The manual microwell EIAs usually require only moderate expertise for the technologist and are completed in 2 to 4 h. These tests, as well as others, are available using automated systems.

ICRs have been developed for antibody detection. Many of these have received FDA clearance and are classified as a waived test according to CLIA. One example of this is HIV-1/-2 antibody detection. Several ICRs are available for this testing. The ICR mechanism for two of these was described earlier in the ICR section. These assays have been reported to perform comparably to laboratory-based automated and manual mirowell EIAs when performed by skilled individuals (Bulterys et al., 2004).

An ICR for detection of infectious mononucleosis heterophile antibodies (ImmunoCard Mono; Meridian Diagnostics, Cincinnati, OH) showed similar sensitivity to latex agglutination and one traditional absorbed erythrocyte agglutination test (Rogers et al., 1999). Other ICRs for antibody detection are being developed.

IgM- and IgA-Specific EIAs

By changing the specificity of the enzyme-labeled component of the EIA test, an IgG-specific assay can be modified for detection of IgM or IgA. Anti-human IgM conjugate will detect IgM, and anti-human IgA will detect IgA. However, this modification alone is usually not sufficient for IgM detection. IgM is present in serum in small quantities compared to IgG, and IgG has been shown to interfere in IgM detection. This interference, as well as IgM-specific testing methods, is discussed in this volume in chapter 11 of this volume, entitled "IgM Determinations."

REPORTING AND QUALITY CONTROL FOR EIAs, OIAs, AND ICRs

Membrane-based EIAs, OIAs, and ICRs, are predictably qualitative in nature, with results reported as positive or negative. However, because many EIAs of microwell-based or other formats have a spectrophotometric readout that is converted to a numerical value (continuous scale), it is possible to report results in either a qualitative (positive versus negative) or quantitative format.

Qualitative Reporting

This is the most basic type of assessment. For membrane-based EIAs, OIAs, or ICR tests that do not yield numerical continuous-scale results and are interpreted simply as positive or negative, translating the observed result into a report is straightforward. If a line develops or a color change is observed in the test area indicating that the analyte had been detected, the result is reported as positive. Otherwise, the result is negative. For EIA methods yielding continuous-scale numerical readings, the reactivity level of the test sample is compared with a predetermined cutoff point. If the sample meets or exceeds the cutoff value, the report of reactive, positive, or perhaps, antibody (or antigen) detected is made. If the reactivity level of the test sample fails to reach the cutoff value, the report for the sample is nonreactive, negative, or perhaps, antibody (or antigen) not detected.

Quantitation for Single Samples

Quantitation for most EIAs remains unique to the assay as specified by the manufacturer. There is little standardization among assays. The actual absorbance reading (or appropriate numerical score) for the test samples may be reported, along with the absorbance value or score that represents the cutoff

value for the test method. By comparing the magnitude of the sample value to that of the cutoff value, the physician can estimate the level of antibody. If the sample value falls very near the cutoff value, the sample has a low level of antibody. Because actual absorbance readings are presented, the numbers are largely meaningless unless the cutoff value is reported. In systems in which the absorbance value is converted to some type of unit (e.g., an EIA unit), this value may be reported rather than the absorbance value. However, like the absorbance value, it is of little value quantitatively unless the cutoff value is provided for comparison. Conversion of EIA values to serial dilution titers is discouraged.

When the absorbance reading of the test sample is divided by the cutoff value of the assay, a quotient termed the index value is generated. Because the index value represents a comparison of the test sample's value and the cutoff value, all values can be interpreted by the same criteria: index value of <1.0 = negative test (antibody not detected) and index value of ≥1.0 = positive test (antibody detected). The magnitude of the index value correlates with the quantity of antibody. Some manufacturers use terms other than index value for this quotient, but the same criteria apply.

Comparison of Levels in Paired (Acute- versus Convalescent-Phase) Samples

Physicians may sometimes rely on determining differences in antibody levels between two samples collected from the patient: one sample is collected as early as possible in the illness (acute phase) and the second is collected 2 to 3 weeks later (convalescent phase). In traditional antibody detection tests performed on twofold serial dilutions, endpoints that showed a fourfold or greater difference in antibody level between the acute- and convalescent-phase samples indicated current or very recent infection. This is an accepted measurement when comparing titers from serial dilutions. Because EIAs are reported in continuous-scale numbers rather than in dilutions, new rules must be supplied by manufacturers of EIA test products to allow significant from insignificant differences in acute- and convalescent-phase antibody levels to be determined.

One manufacturer suggests that a quotient (convalescent-phase index value divided by acute-phase index value) of ≥1.9 indicates a significant difference in antibody level for some types of antibodies (Diamedix Corp., Miami, FL). However, the necessary quotient for significance, even with products produced by the same manufacturer, may vary according to the type of antibody being measured (e.g., the criteria for significant difference in levels of rubella antibodies may be different from those for measles antibody). Many manufacturers of EIAs do not define parameters for evaluating the magnitude of difference between two samples.

Unfortunately, serum samples containing standardized levels of antibodies or antigens are not widely available. This makes nation- or world-wide standardization of assays difficult, if not impossible. However, most commercially available EIA products are marketed with calibrator samples that control calibration; for some products, several calibrators are included to generate a curve. Most EIA systems also include both positive and negative serum samples, intended for testing in each run of patient samples. All of these control materials must produce results that fall within acceptable limits with each run of testing if the run is to be considered valid and the patients' results are to be reported. One additional type of control that must be incorporated into EIA testing is a lot-to-lot control that is tested when new lot numbers of reagents are put into service. The purpose of this type of control is to ensure that each new lot of reagents produces results comparable to those produced by the previous lot. This type of control is especially important in tests that provide a quantitative value. Lot-to-lot control materials are not routinely provided by manufacturers and must be purchased or otherwise obtained by the laboratory. Patient samples (or pooled patient materials) previously tested may be used for this purpose. Prior to being put into service, all lot-to-lot control material must be tested in duplicate or replicate in several runs of testing, including more than one lot number of reagents, to define an acceptable range. Most laboratories prefer to use a range that includes ±2 standard deviations, although some laboratories use ±3 standard deviations as their cutoff.

CONCLUSIONS

EIA technology, with its extreme versatility, offers an approach for performing many of the assessments required in the diagnostic virology laboratory. EIA is currently a mainstay in diagnostic testing, and continuing development will undoubtedly produce additional EIA-based assays to replace antiquated technologies and solve contemporary problems. Advances in EIA automation are facilitating more rapid and cost-effective viral antibody and antigen testing. The application of membrane EIAs, OIAs, and ICRs to viral antigen and antibody detection have opened an avenue for rapid viral diagnosis that allows testing to be conducted in more venues such as point-of-care and physician's office laboratories. Application of all of these technologies is enhancing the availability and timeliness of viral diagnostic laboratory results.

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Immunoenzymatic Techniques for Detection of Viral Antigens in Cells and Tissue

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8

Since their introduction, immunoenzymatic techniques for the detection of viral antigens have served as important tools to detect, confirm, and identify viruses from direct specimen, cell culture, and tissue. Although applications for immunoenzymatic techniques (i.e., immunoperoxidase or immunohistochemistry [IHC]) have become more limited, owing in large part to the widespread use of enzyme immunoassay and immunofluorescence, IHC has undergone significant evolution and refinement, offering histopathologists and laboratorians a sensitive and accurate method to diagnose viral infections. Similar to enzyme immunoassay and immunofluorescence, IHC does not rely on differential cytopathic effect (CPE) for identification This feature enables the detection of viruses that do not produce characteristic CPE or are difficult to cultivate (Tamm et al., 2001; Chemaly et al., 2004), which greatly improves turnaround time and test sensitivity (Benjamin and Gray, 1974; Benjamin, 1977; Cevenini et al., 1983). Furthermore, despite advances in molecular testing, application of IHC to tissue specimens (e.g., formalin-fixed, paraffin-embedded tissue sections) with concomitant morphologic examination continues to serve as the gold standard for diagnosis and confirmation of many viral infections (Ljungman et al., 2002). In this chapter, we review the developments in IHC techniques, with particular emphasis on antibody preparations, pretreatment antigen retrieval (AR), and state-of-the-art enzymatic signal amplification methods. We also discuss potential applications for these techniques, focusing on their optimal use by clinical laboratories and histopathologists for particular clinical contexts and viruses.

HISTORY

The use of labeled antibodies for the detection of infectious agents in tissue was first demonstrated in 1942, with identification of pneumococcal antigens by direct fluorescent antibody in the livers and spleens of experimentally infected mice (Coons et al., 1942). These methods were subsequently applied to detect other bacteria and viruses in vivo and in vitro but were limited by obscuring background autofluorescence, lack of permanence, and requirement of fluorescent microscopy (Coons et al., 1950; Kaplan et al., 1950). In the 1960s, it was observed that replacement of fluorescent labels by an enzyme allowed deposition of a precipitate at the site of antibody-antigen binding that was permanent and visible for light microscopic examination (Nakane and Pierce, 1966, 1967). This immunoenzymatic method involved: (i) reaction of tissue antigens with primary antibody (unlabeled rabbit antibodies), (ii) incubation with horseradish peroxidase-conjugated anti-rabbit sheep immunoglobulin, and (iii) visualization of antibody complex by exposing the tissue to a solution containing an appropriate electron transfer substrate and electron acceptor or chromogen, such as hydrogen peroxide (H_2O_2) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Nakane and Pierce, 1967). In this method, bound enzyme catalyzed oxidation of H_2O_2 and reduction of DAB, resulting in deposition of a nondiffusible, insoluble dark brown precipitate permanently localized to the site of the target antigen.

Since that time, the basic principles of immunoenzymatic methods have been maintained, but advances in technique and reagents have led to substantial improvements in performance and interpretability. Nonspecific staining due to endogenous tissue peroxidase activity has been minimized through pretreatment with peroxidase-inactivating reagents (e.g., H_2O_2) (Straus, 1971; Pitts and Williams, 1974) or use of alternative enzymes (e.g., alkaline phosphatase). Simultaneously, the development of monoclonal antibody reagents and incorporation of tissue blocking solutions (e.g., nonimmune serum) has reduced nonspecific antibody binding. Innovations in detection and signal amplification have dramatically increased test sensitivity. Indirect methods of antigen-antibody labeling originally developed to avoid diminishing primary antibody affinity by attachment of large conjugates (Weller and Coons, 1954) introduced the concept of signal amplification. Subsequent methods utilized elaborate bridging antibodies and antibodies with specificity for detection enzymes (e.g., peroxidase-antiperoxidase [PAP] methods) as a means of attaching more enzyme moieties (Sternberger et al., 1970; Burns, 1975). Such methods were eventually replaced by detection systems based on the high affinity of avidin/streptavidin for biotin (e.g., avidin-biotin complex, the enzyme, and labeled streptavidin-biotin [LSAB]), which increased enzyme attachment, and also were easier to use (Nagle et al., 1983; Giorno, 1984; Shi

et al., 1988; Milde et al., 1989). Commercial kits utilizing biotinylated antibodies and LSAB remain in widespread use today. Most recently, "polymeric" techniques, including binding enzyme moieties to dextran backbones (Zhang et al., 2000; Banks et al., 2003) or micropolymers (Ramos-Vara and Miller, 2006), have become available. In addition, the catalyzed signal amplification (CSA) method, which utilizes repetitive enzymatic precipitation steps (Speel et al., 1999; Hashizume et al., 2001), is another alternative technique that has greatly enhanced test sensitivity. In general, the relative sensitivity of the various methods increases as follows: direct < indirect < PAP, avidin-biotin complex < LSAB < polymeric methods < CSA (Mokry, 1996).

Improvements in tissue processing, development of prestaining treatments, and use of automated immunostainers have led to additional increases in the sensitivity and reliability of target antigen detection (Curran and Gregory, 1978; Taylor, 2000; Hashizume et al., 2001; Shi et al., 2001). The choice of fixative (e.g., formalin or alcohol) and length of fixation have been recognized to potentially diminish subsequent antigen binding and detection when fixation-induced cross-linking of proteins and cellular constituents obscures antigenic epitopes. Prestaining procedures (referred to as AR methods), such as timed proteolytic digestion (e.g., trypsin) or application of heat in aqueous solution via microwave or pressure cooker, have been developed to recover alterations in antigenic structure occurring during the fixation process (Shi et al., 2001). Heat-induced AR with or without a component of proteolytic digestion is now the most commonly used pretreatment method. Lastly, the routine use of automated immunostainers has rendered the IHC method more attractive by reducing time and labor while improving stain quality and consistency (Taylor, 2000; Hashizume et al., 2001).

METHODS AND LIMITATIONS

Modern IHC stains are exquisitely sensitive and can detect viral proteins in the absence of well-defined inclusions. However, many variables impact test performance (Taylor, 2000; Shi et al., 2001; Nuovo, 2006a). For viruses not actively replicating or producing significant target protein, IHC will have limited utility (Nuovo, 2006a). Each monoclonal antibody will have differing affinities and specificities for specific viral proteins and may not detect all virus subtypes. Sampling error, timing of fixation relative to specimen collection, type of fixative, use of blocking reagents, AR, and antibody concentration all affect overall sensitivity and specificity (Taylor, 2000; Nuovo, 2006a). The availability of high-quality commercial kits utilizing PAP, LSAB, or polymeric or CSA methods has reduced but not eliminated these test variables.

The selection of a specific IHC method (e.g., commercial kit) is often a matter of personal preference because practical differences in the performance of most modern commercial kits are relatively small. However, the intended use and target should be considered. Cost, ease of use, and compatibility with laboratory workflow or automated stainer are important considerations. In our laboratory, we have had good experience with a simple and inexpensive direct immunoperoxidase method for the detection and confirmation of herpes simplex virus (HSV) from cell culture (Crist et al., 2004). However, for formalin-fixed tissue, we use a combination of LSAB and polymeric and CSA methods on an automated stainer.

VARIABLES AFFECTING TEST PERFORMANCE

Fixation

Proper fixation of tissue prior to examination is necessary to maintain tissue architecture, preserve antigenicity, and prevent degradation. As stated previously, the fixation process may create chemical cross-linkages that alter three-dimensional protein structure and epitopes, which can compromise the detection of antigen by the target antibody (Shi et al., 2001). Not all epitopes are equally affected, but prior to any systematic application of IHC, evaluation of various combinations of fixative, fixation time, and antibody with and without AR may be desirable. In practice, formalin remains the predominant general purpose fixative in use.

Antibodies

A wide array of polyclonal and monoclonal antibodies are now commercially available for the detection of viral and other protein targets. Few antibodies are U.S. Food and Drug Administration approved or labeled for in vitro diagnostic use. The most common clinically relevant virus antibodies are available only in analyte-specific reagent or research-use-only format. Epitope specificity and affinity of antibodies vary among vendors. Specificity is generally reliable across manufacturer lots, but optimal titer may need to be adjusted. In general, monoclonal reagents are more specific and predictable in their antigenic reactivity than polyclonal antibodies. The performance characteristics of monoclonal antibodies may vary with type of fixative, degree of antigen exposure, and virus subtype. Preprepared blends (mixtures) of monoclonal antibodies have been developed to improve their ability to capture various virus subtypes with the goal of optimizing test sensitivity and retaining specificity.

Blocking

Traditionally, normal, nonimmune serum has been used to block nonspecific tissue binding prior to incubation with primary antibodies. Today, many primary antibody reagents include nonimmune serum, making this step often unnecessary. Blocking of endogenous peroxidase with 3% H_2O_2 is routinely included when horseradish peroxidase enzyme chemistry is utilized. Biotin-blocking reagents may be indicated when staining tissues with high levels of endogenous biotin (e.g., liver or kidney) with avidin or streptavidin-biotin detection systems (Wood and Warnke, 1981).

AR

AR refers to a process of tissue treatment that may be necessary or beneficial prior to IHC staining to restore or optimize antigenicity following fixation. As discussed above, fixation-induced changes may mask target epitopes, and while AR is not necessary in all cases, antigenicity can be improved or restored through controlled exposure to heat and/or proteolytic digestion (Curran and Gregory, 1978; Shi et al., 2001). The pH of the retrieval solution is also important and may be adjusted. When investigating any new antibody and stain, different AR combinations (e.g., high heat, low heat, or proteolytic digestion) should be compared by performing a battery of tests, including fixed and unfixed tissue stained with and without AR. Knowledge of the location of the target protein (e.g., membrane versus nuclear) is important to assess staining results and may alter the AR approach. A more comprehensive discussion of this topic has been published (Shi et al., 2001).

Assay Optimization

To minimize nonspecific background staining and determine the need for AR, reagents for each staining protocol must be optimized. For all reagents, test performance should be evaluated by individual laboratories prior to implementation for diagnostic use. Multiple dilutions of primary antibody should be compared to determine the optimal titer for the staining method, fixative, and AR combination. The optimal titer of antibody should be verified when a new lot of antibody reagent is put into use. Additionally, DAB and H_2O_2 concentrations may be titrated for faster or slower substrate conversion to meet the needs of a particular system or user. (Note, DAB is a suspected carcinogen, and care must be taken when handling and disposing of this material.)

Quality Control and Standardization

As with all laboratory testing, quality control is integral to the reliable and reproducible performance of IHC as a diagnostic tool for the detection of infectious organisms (NCCLS, 1999). Quality control of IHC staining should routinely include three controls, (i) a positive tissue section prepared and stained as a patient tissue sample, (ii) a negative tissue section prepared and stained as a patient sample with omission of primary antibody, and (iii) an internal negative control (unstained portions of patient tissue sample), and the assessment of appropriate control reactivity prior to interpretation and result reporting for the patient sample. Some practitioners recommend that negative tissue controls should be of the same organ or type as the sample tissue (background staining may vary between tissues) and that nonpositivity should be verified prior to use (e.g., by in situ PCR) (Nuovo, 2006a). Others have raised concern that the use of controls is not sufficient for monitoring performance and that standardization of the complete process (i.e., collection, fixation, processing, AR, staining, and interpretation) is necessary (Taylor, 2000). Clinical Laboratory Standards Institute guidelines for the performance of IHC were created in 1999 and proficiency materials are now available (NCCLS, 1999; Taylor, 2000). Automation of staining and the development of standardized methods including quantitation may also help, but the lack of uniform control materials (e.g., positive tissue) remains a barrier to ensuring optimal test performance.

APPLICATIONS

The unique performance characteristics of IHC make this method practical and relevant for many applications. The method is independent of production of CPE or typical viral inclusions, generates permanent preparations that can be viewed with an ordinary light microscope, and allows simultaneous evaluation of stained and unstained cells in tissue sections (Table 1). These strengths offer a wide array of applications that can greatly benefit the practices of histopathology and clinical virology. In the following sections, general guidelines for the interpretation of IHC and its applications are discussed with regard to type of specimen and virus, highlighting specific clinical situations where this method may prove most valuable as a diagnostic tool.

Guidelines for Interpretation

Technologists and histopathologists should familiarize themselves with the diversity of staining patterns when interpreting IHC stains. Knowledge of expected localization of staining (e.g., type of cell and nuclear, cytoplasmic, or membranous) should be used as internal verification of antibody specificity and performance. Nonspecific staining due to antibody trapping around defects or edges of culture or tissue sections and endogenous peroxidase activity (e.g., neutrophils and plasma cells) in inflamed tissues or specimens can be sources of false reactivity. In evaluating stained histologic sections, it is important to verify that the region of interest is represented in the stained section. Once specificity of staining is confirmed, the presence of any positive staining for virus in direct specimen, cell culture, or tissue is generally considered significant and, for tissue, usually diagnostic of active infection (see below). For tissue sections, the degree and nature of host response (e.g., inflammation) should be assessed to further confirm IHC results. Despite the absence of standardized methods for quantitation, the proportion of infected cells offers a semiquantitative indication of viral activity (Nuovo, 2006a) that can be useful in select clinical contexts, such as determining response to antiviral therapy.

TABLE 1 Strengths and weaknesses of immunoenzymatic methods for antigen detection

Strengths
Applicable to most protein-productive viruses
Suitable for direct specimens, culture, or fixed tissue
Highly sensitive for certain clinical contexts
Highly specific
Easy to perform and use
Permanent
Suitable for light microscopy
Allows simultaneous evaluation of stained and nonstaining cells
Available in commercial kit form
Easily automatable
Weaknesses
Susceptible to nonspecific staining due to endogenous enzyme or avidin binding activity
Insensitive to latent or non-protein-productive viral infection
Less sensitive than NAAT methods for select clinical syndromes

Detection from Clinical Specimens

While largely supplanted by immunofluorescent methods in clinical virology laboratories, immunoperoxidase methods compare favorably and have been used to successfully detect most clinically relevant viruses directly from clinical material (e.g., nasopharyngeal washes or mucocutaneous lesions) and peripheral blood mononuclear cells (Benjamin, 1977; Moseley et al., 1981; Cevenini et al., 1983). The importance of an experienced technologist in reading these preparations cannot be overemphasized. The less-experienced technologist will frequently misinterpret the presence of cells with endogenous peroxidase activity (e.g., neutrophils) in direct specimens and compromise test specificity (Gardner et al., 1978).

Detection from Cell Culture

Immunoperoxidase methods can shorten the time needed to detect viral growth in culture (e.g., cytomegalovirus [CMV], HSV, and myxo- and paramyxoviruses) (Miller and Howell, 1983) with performance comparable to both immunofluorescent detection (Hahon et al., 1975; Schmidt et al., 1983)

and commercial, genetically engineered enzyme-linked detection methods (Crist et al., 2004). Advantages of immunoperoxidase staining include permanence and lack of requirement for fluorescent microscopy.

Detection of Viruses from Tissue

The development and optimization of IHC for the detection of protein antigens in formalin-fixed, paraffin-embedded tissue sections has revolutionized practices in all areas of histopathology (Mukai and Rosai, 1981; Taylor, 2000). In this setting, IHC has become a routine component of histologic examination, facilitating the detection and confirmation of many more viral infections than traditionally stained sections or reliance on the presence of viral inclusions alone (Tamm et al., 2001; Nuovo, 2006a, 2006b). For viruses associated with characteristic inclusions, IHC is more sensitive than traditional techniques because it can identify the putative virus from atypically infected cells or cells without well-developed inclusions (Arbustini et al., 1992, 1996; Colina et al., 1995; Tamm et al., 2001; Chemaly et al., 2004).

Despite advances in molecular techniques, demonstration of an infectious agent (e.g., virus) within tissue in the setting of host injury or immune response remains the gold standard for diagnosis and/or confirmation of many viral infections (e.g., CMV) (Ljungman et al., 2002). Nucleic acid amplification tests, when performed from plasma or blood, will miss localized infections without viremia (Ketteler et al., 2000; van Burik et al., 2001; Jang et al., 2004; Mori et al., 2004; De Bartolomeis et al., 2005) or, when positive, may not always correlate with invasive viral disease (Roberts et al., 1998; Zedtwitz-Liebenstein et al., 2004). Occasionally, formalin-fixed tissue may be the only specimen available at the time a viral etiology is considered or recognized (Cioc and Nuovo, 2002; Nuovo, 2006a). For such cases, classical histological examination for viral infection combined with techniques such as IHC, in situ hybridization (ISH), or in situ polymerase chain reaction (PCR) may allow confirmation of viral diagnosis. ISH and in situ PCR have characteristics that may be preferred over IHC for some viruses and clinical contexts such as the detection of viruses in the latent state or for clinical syndromes where viruses are present in low copy numbers (e.g., human papillomavirus, human immunodeficiency virus, hepatitis C virus) (Nuovo, 2006a, 2006b).

Overall, IHC serves as an important diagnostic tool for (i) detection of viral protein from actively replicating productive viruses (e.g., CMV, HSV, adenovirus, some RNA viruses), (ii) detection of viruses when only formalin-fixed tissue is available, (iii) correlating results from nucleic acid amplification testing (e.g., localized CMV infection), (iv) detection of unusual viruses for which detection by routine laboratory methods is not reasonable or available (e.g., dengue), and (v) clinical or research contexts where histologic confirmation is necessary.

DNA Viruses

CMV

Detection and confirmation of CMV infection in tissue remains one of the most common viral indications for performance of IHC stain. As a common herpesvirus with a well-recognized latent state, it has been observed that qualitative nucleic acid amplification tests (NAAT) and, occasionally, viral culture of bronchoalveolar lavage fluid, urine, and blood can be positive in the absence of clinically apparent CMV disease (Ljungman et al., 2002). NAAT detection of CMV from tissue in the absence of IHC or morphologic confirmation of infection has been attributed to detection of CMV DNA in the latent state or in passenger leukocytes (Pucci et al., 1994; Hendrix et al., 1997; Ljungman et al., 2002). Thus, histologic demonstration of CMV infection remains both an important diagnostic tool to distinguish CMV infection from active disease and an important criterion in diagnostic guidelines (Ljungman et al., 2002). In certain clinical contexts such as CMV gastrointestinal disease, NAAT from blood may be negative in the presence of clinically significant end-organ disease, but infection can be confirmed by IHC staining of a tissue biopsy specimen. In summary, IHC for CMV in tissue is useful to distinguish active disease from latent or clinically insignificant infection and remains important in the diagnosis of localized end-organ disease when antigenemia or viral DNAemia is absent.

HSV

The performance of IHC for detection of HSV from tissue is similar to that of IHC for CMV. Most HSV-associated central nervous system clinical syndromes (e.g., meningoencephalitis and retinitis) are best diagnosed with NAAT; however, IHC for HSV can prove useful when an etiologic diagnosis has not been established and only formalin-fixed tissue is available (e.g., a brain biopsy specimen from unexplained meningoencephalitis, postmortem examination of fetal demise, or a liver biopsy specimen for hepatitis). In such settings, IHC testing for HSV is generally performed as part of a battery of tests (e.g., IHC, ISH, or in situ PCR) in an effort to confirm a diagnosis (Cioc and Nuovo, 2002). IHC is also helpful in distinguishing HSV from varicella-zoster virus (e.g., skin biopsy specimen) infection when culture or NAAT is not available.

Other DNA Viruses

Detection of human herpesvirus 8 in tissue by IHC has been suggested as an adjunct to distinguish Kaposi's sarcoma from other benign or malignant vascular tumors with which human herpesvirus 8 is not associated (Patel et al., 2004). Epstein-Barr virus can occasionally be detected by IHC but is better detected by ISH unless active viral replication is present. Adenovirus is readily detectable by IHC from tissue with performance similar to that for CMV and HSV and may be useful in some clinical situations. For example, IHC staining of tissue may be helpful to distinguish adenovirus infection from adenoviral-like cytologic changes which can be observed with nonviral reactive or reparative changes. Histopathologists should also be aware that IHC staining for adenovirus may rarely be falsely negative if the antibody clone does not capture all adenoviral serotypes. Because of its latency, human papillomavirus infection is not reliably detected by IHC (Nuovo, 2006a). IHC has been used to confirm the etiology of cytologic changes of BK virus in renal transplant biopsy specimens, hepatitis B virus in routine liver biopsy specimens, and parvovirus in bone marrow, but the performance of these stains is not well characterized.

RNA Viruses

Limited data exist that compare the sensitivity of IHC methods with laboratory culture or PCR for the detection of RNA viruses. Among respiratory viruses, IHC performs well for detection and confirmation of respiratory syncytial virus and measles (Nuovo, 2006a). It remains a confirmatory

method for diagnosis of rabies from brain or skin biopsy when performed together with NAAT, which is more sensitive. IHC staining of coxsackievirus from myocardium or placenta has been used to determine the etiology in cases of unexplained sudden death or fetal demise (Cioc and Nuovo, 2002; Nuovo, 2006a; Nuovo, 2006b). Similarly, rotavirus has been detected by IHC in endothelial cells of the brain and heart in rare cases of sudden unexplained death (Cioc and Nuovo, 2002). Lastly, IHC has been an important tool for confirming the presence of rare or uncultivable RNA viruses that cause Rift Valley fever, yellow fever, and dengue fever. IHC is not helpful in the detection of RNA viruses with a prolonged latent state (e.g., human immunodeficiency virus) or low level of antigenic protein production (e.g., hepatitis C virus) (Nuovo, 2006a).

Detection of Multiple Antigens

One recent development of interest has been the ability to simultaneously stain tissue sections for more than one antigen. As such, these techniques have been used to assess localized cytokine production (e.g., tumor necrosis factor alpha, interleukin-2, and interleukin-8) in relationship to viral infection as an aid in understanding the pathogenesis of AIDS dementia, rabies encephalitis, and dengue fever (Nuovo and Alfieri, 1996; Nuovo et al., 2005; de Macedo et al., 2006). In select cases (e.g., unexplained meningoencephalitis, intrauterine fetal demise), staining for cytokines with or without concomitant viral staining may be useful in screening for occult viral infection where absence of cytokine production has promising negative predictive value (Nuovo, 2006a).

FUTURE DIRECTIONS

Immunoenzymatic staining methods have undergone tremendous evolution in the last two decades, offering a diagnostic alternative to immunofluorescent methods to detect viruses directly from patient specimens or culture. More significantly, the application of these stains to detect viruses from tissue sections is a valuable technique, providing information complementary to viral culture and NAAT results. Improvements in signal amplification combined with AR, reduction in nonspecific background staining, and development of automated stainers have greatly enhanced test performance and usability. In their current formulations, IHC sensitivities now approach the performance of in situ PCR from tissue for some protein-productive DNA viruses (e.g., CMV, HSV, varicella-zoster virus, adenovirus, hepatitis B virus) and RNA viruses (e.g., measles, respiratory syncytial, dengue). When compared with viral culture or NAAT, IHC is generally less sensitive, but in certain clinical contexts, IHC offers superior specificity and remains the preferred diagnostic test. In summary, communication between the histopathologist, clinical virologist, and clinician is essential to optimize the application of IHC methods to provide accurate and clinically meaningful results that will enhance our ability to diagnose viral infections and directly impact patient management.

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Neutralization

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9

The neutralization test has been used in virology longer than any other serologic procedure. Despite its relative antiquity, neutralization techniques remain relevant today and remain one of the most specific serologic assays available.

Neutralization of a virus is defined as loss of infectivity through reaction of the virus with specific antibody. To test for virus neutralization, virus and serum are mixed together, incubated under appropriate conditions, and then inoculated into a susceptible living host for detection of nonneutralized virus. Nonneutralized virus is detected by looking for viral growth, using indicators such as cytopathic effect (CPE), plaque formation, or metabolic inhibition (Schmidt, 1979).

Neutralization assays can be used to identify a virus or to determine the specific antibody response to a viral infection. Identification of a virus isolate is performed by neutralization of an appropriate infectious dose of that virus by a well-standardized specific immune serum. A fourfold rise (using twofold dilutions) in host neutralizing antibody titer between acute- and convalescent-phase sera to a standardized virus inoculum is diagnostic for recent infection. The presence of neutralizing antibody in a single serum sample indicates previous infection or exposure and may be used as a measure of successful immunization or immunity. Because this test is based on immunologic specificity, the neutralization test is used for differentiating antibody response between closely related viruses (e.g., among various arboviruses) and serves as the gold standard against which the specificity of other serologic procedures are evaluated. In addition to their use for diagnostic purposes, neutralization assays have many research applications, such as antigenic mapping for neutralizing epitopes and determining the mechanism of viral neutralization (Crawford-Miksza et al., 1999). Neutralizing monoclonal antibodies have been described and used, for instance, to study the mechanism of antibody-mediated viral neutralization (Varghese et al., 2004).

While the basic principles of performing the neutralization test remain unchanged over the past three decades, important innovations have occurred that improve efficiency, timeliness, and sensitivity. Micro methods, performed on a 96-well plate or other multiwell formats, result in increased throughput and reduce the need for large volumes of sera and reagents (Schmidt, 1979). The use of genetically altered viruses that express markers such as green fluorescent protein (GFP) or luciferase enzyme to measure infectivity provide increased sensitivity, objectivity, and rapid interpretation of results (Khawplod et al., 2005; Kolokoltsov et al., 2006; Wang et al., 2004). Automated plaque counting enables more rapid, reliable, and efficient measurements of viral growth (Zielinska et al., 2005). The use of pseudovirus, which expresses the surface proteins of one virus and the core of another virus, allows for improved biosafety when working with high-risk agents (Bartosch et al., 2003; Hang et al., 2004; Kolokoltsov et al., 2006).

TEST MATERIALS AND REAGENTS

The use of standardized components is critical to performing neutralization assays. For viral identification, well-characterized pretitered antiserum or well-standardized immune serum pools are used. Similarly, to measure antibody response to a virus, a well-characterized, pretitered virus is required. Finally, to monitor for viral inactivation (neutralization), a living host system is required.

Virus

Virus Preparation

Virus can be prepared in-house from extracts of infected cell cultures, homogenates from animal tissues or components of embryonated eggs. Virus can also be purchased commercially. Virus is propagated by inoculating a susceptible host system with either stock virus or the viral genome in the form of a plasmid. The virus is harvested after allowing for optimal propagation. With a few exceptions, cell cultures are the ideal host system used for viral cultivation, and they may be prepared in house or purchased commercially. Cells prepared using in-house systems offer the advantages of maximum quality control and consistent availability. Mouse inoculation is used to prepare high-titered stocks of some enteroviruses, arboviruses, and rabies viruses. Similarly, high-titered strains of influenza virus are best prepared in embryonated eggs. Regardless of the system used for cultivation, the virus should always be titrated in the host system that will be used for the neutralization test.

Assessing Viral Growth and Titration

For viruses that readily produce a rapid and complete CPE in cell cultures, such as the enteroviruses, adenoviruses, or herpes simplex virus, growth and titration are monitored by scoring CPE. This type of titration is known as a quantal

assay because instead of counting the number of infectious particles in the inoculum, the number of infected tubes, wells, or animals is scored. A single infectious event is sufficient to produce a positive result throughout the host system. Thus, each inoculated host is scored as positive or negative based on the presence or absence of infectious particles.

The second method for measuring growth and titration is quantitative and involves counting of infectious particles. An example is the plaque assay (Schmidt, 1979). Viruses, both those that readily produce CPE and those that are less cytopathic, may still produce plaques. In the plaque assay, inoculated cell culture systems are overlaid with a semisolid gel to prevent the spread of progeny virions from infected cells to distant cells. Each infection gives rise to a localized focus of infected cells. This focus of infected cells, or single plaque-forming unit (PFU), may be visualized by fixing the monolayer and staining with a vital dye (e.g., neutral red). The PFU will be observed as an absence of cells, i.e. a plaque, for cytopathic viruses. For viruses that are not cytopathic, the monolayer can be fixed and then stained with a virus-specific antibody labeled with enzyme or fluorescent dye to enable the plaque to be visible by direct microscopic observation.

Determining Viral Titer

To titrate stock virus or an isolate, serial 10-fold dilutions are prepared in maintenance medium and inoculated into a susceptible host system with fixed volumes of each dilution (e.g., 50 µl/well, 4 wells each for each 10-fold dilution is delivered to a 96-well cell culture plate). The cells may be in a monolayer, or a fixed number of cells may be added to each well after the virus is added. The host system is then monitored for signs of viral replication. In a quantal assay, the endpoint is the reciprocal of the highest dilution of virus that infects 50% of the host system. This endpoint dilution is determined by the Reed-Muench method and contains 1 50% tissue culture infective dose ($TCID_{50}$) of virus per unit of volume, or the amount of virus that will infect 50% of the cell cultures inoculated (Table 1). The concentration of virus generally used in the neutralization test is 100 $TCID_{50}$ or 100 50% lethal doses per unit of volume (Fig. 1). The titer may be determined more exactly by titrating 0.5 $\log_{10}$ dilutions of the virus.

Quantitative assays, such as the plaque assay, are scored by counting the number of plaques, with one plaque being the result of one infectious event or virion. To determine a PFU titer, serial 10-fold dilutions of the virus are prepared and added to a cell monolayer. The plate is overlaid with a semisolid gel and incubated until plaques develop. Plaques can be visualized by fixing cells and staining with a vital dye, antibodies labeled with fluorescent dye, or an enzyme. The plaques are counted, and the titer is determined. For instance, if the plaques overlap or are too numerous to count at a titer of 10^{-4}, 50 PFUs are counted at 10^{-5} and 2 at 10^{-6}; the titer of this virus is 50 PFUs per unit volume at 10^{-5} dilution. Plaque titrations provide a quantitative value for the number of infectious particles in a viral preparation and are generally considered a more accurate estimate of the viral infectious titer. The challenge dose for a plaque reduction neutralization test is preset. The neutralization endpoint for the plaque reduction neutralization test is set as a percent decrease in the plaque number, with 50 or 90% reduction frequently serving as the endpoint.

In cases where the virus grows very slowly or does not produce visible plaques, staining with virus-specific antibody labeled with a fluorescent dye or enzyme is used to visualize plaques. For example, rabies is a virus that produces little or no CPE in cell culture and for which plaques cannot be easily viewed. The rabies fluorescent focus inhibition test is a rapid, sensitive neutralization assay in which foci of rabies virus-infected cells are stained with virus-specific antibodies (Hooper, 2002). Human cytomegalovirus (CMV) grows extremely slowly in cell culture, and scoring neutralization by CPE requires a minimum of 5 to 10 days. The use of monoclonal antibodies (MAb) to CMV early nuclear protein allows CMV neutralization test results to be obtained in 24 h (Braun and Schacherer, 1988).

Colorimetric assays that measure the amount of cell destruction or changes in the pH of the media (metabolic inhibition assay) are alternatives to scoring viral CPE or counting PFUs (Schmidt, 1979). In the colorimetric assay, virus is titrated in 96-well plates and equal volumes of diluent are added to control wells. The cells are trypsinized and counted, and a predetermined cell number is added to each well. Plates are incubated in 5% CO_2 and then fixed and stained with neutral red. The amount of neutral red absorbed

TABLE 1. Calculation of 50% mortality (virus titration) with sample data[a]

Virus dilution	No. of deaths/no. inoculated	Cumulative no of:		Mortality ratio	% Mortality
		Deaths	Survivors		
10^{-4} ($1:10^4$)	5/5	10	0	10/10	100
10^{-5} ($1:10^5$)	4/5	5	1	5/6	83
10^{-6} ($1:10^6$)	1/5	1	5	1/6	17
10^{-7} ($1:10^7$)	0/5	0	10	0/10	0

[a]Interpolation formula: $\frac{\%\text{ mortality greater than } 50\% - 50\%}{\%\text{ mortality greater than } 50\% - \%\text{ mortality less than } 50\%}$

Substituting: $\frac{83 - 50}{83 - 17}$ $\frac{33}{66}$

a. Multiply the interpolative value by the negative $\log_{10}$ of the dilution ratio.
Negative $\log_{10}$ of the dilution ratio = –1
Interpolative value = 0.5
Corrected interpolative value = –0.5
b. The endpoint dilution associated with 50% mortality is located between the 10^{-3} and 10^{-6} dilutions.
c. The $\log_{10}$ of the 50% endpoint dilution is estimated by adding the correct interpolative value to the $\log_{10}$ of the dilution above 50% – 5 + (–0.5) – –5.5.
d. The 50% endpoint dilution is estimated at 10^{-55}.
e. The 50% titer is estimated at 10^{55}.

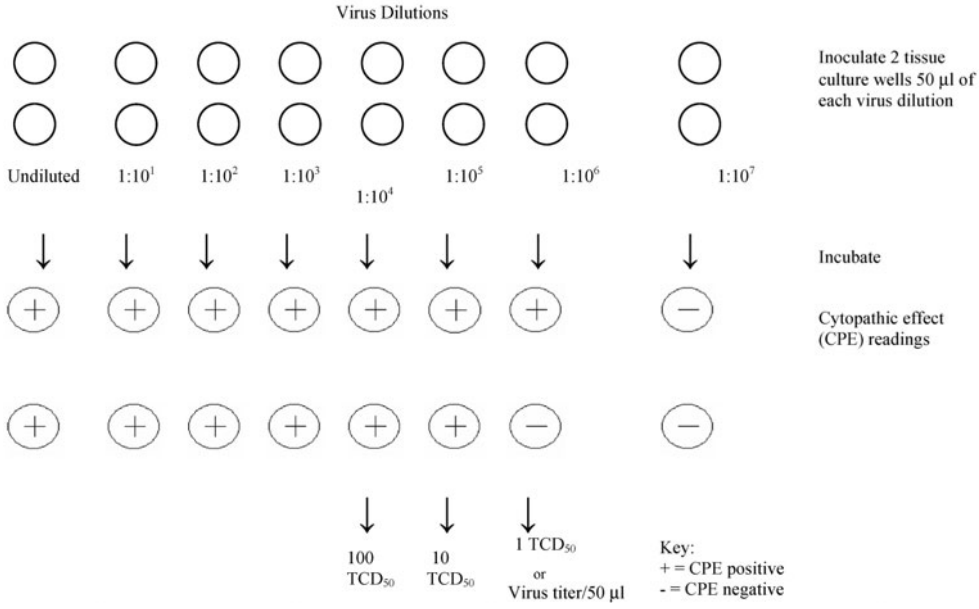

FIGURE 1 This virus titration demonstrates that the endpoint titer is the highest dilution of virus that infects 50% of the inoculated cell cultures.

by intact cells as read by a plate reader is directly proportional to the number of healthy cells. The viral titer, calculated as 50% neutral red absorbance compared to the cell control, correlates well with the virus titer determined by CPE (Crawford-Miksza and Schnurr, 1994).

Determining if the virus was inactivated or grew in the presence or absence of test sera is the crucial step of the neutralization assay. A number of advances that provide more rapid and objective methods for determination of viral titers have been developed. These include labeling the virus with reporter genes or molecules such as GFP or luciferase, which are expressed when the virus genome is transcribed and translated. GFP acts as a surrogate marker for viral growth and is read using a UV microplate reader or UV light or by fluorescence microscopy. Luciferase is an enzyme that acts on a substrate to produce a luminescent product when translated. Replication of a recombinant virus is standardized by determining the titer of the recombinant virus in parallel with the parental virus and comparing the expression of GFP or luciferase with a standard measure of viral multiplication such as PFUs or CPE. The recombinant virus is then calibrated so that its infectious titer is equivalent to that of the parental virus using the standard method for titration.

Another modification that takes advantage of recombinant DNA technology is the creation of pseudoviruses. Pseudoviruses contain surface antigens that are the target of neutralizing antibodies but the core, replication coding region, of another virus. They present a much lower biosafety risk when working with biosafety level 3 and select viral agents than the original, wild-type virus. The pseudovirus core may be replication deficient, making it even safer. An example of a pseudovirus construct is Venezuelan equine encephalitis virus surface proteins on a murine leukemia virus core (Kolokoltsov et al., 2006). When this recombinant virus enters susceptible cells, luciferase is expressed. Luciferase expression as measured by enzymatic activity is linear for 50 h. Comparison of the growth of parental Venezuelan equine encephalitis virus with that of the pseudovirus allows the inoculum of pseudovirus to be standardized so that an amount of pseudovirus equivalent to 100 $TCID_{50}$ of parental virus for use in neutralizing assays is established.

Serum

Specific immune serum can be purchased commercially or prepared by immunizing animals and harvesting the serum. The antiserum must be standardized for use in the neutralization test by titration against both its homologous virus and heterologous virus(es). For example, neutralizing antisera for typing a specific adenovirus serotype should have titers determined against the virus that the host animal was immunized against as well as with other adenovirus serotypes to ensure serotype specificity. The neutralizing titer of the serum is determined by testing a series of twofold dilutions with the specific virus that was the immunizing agent. One antibody (Ab) unit is the highest dilution of the serum neutralizing the virus (Fig. 2.).

When immune sera is prepared, preimmune serum for each animal should be collected prior to inoculation and tested to ensure that the immunized animal had no previous exposure to the specific or related virus(es) of interest. Standardization of immune serum requires that the serum be tested in the neutralization assay against the virus used to immunize the animal and closely related viruses. It should have a high titer against the virus used to immunize and minimal or no titer against related viruses.

MAbs are a reproducible source of highly specific reagents. MAbs made to whole virus or viral proteins may be neutralizing or nonneutralizing. The neutralization capacity of an antiviral MAb is determined by titration against the virus of interest. Serial dilutions of the MAb are mixed with the virus, incubated, inoculated into the appropriate host system, and observed for neutralizing potency (i.e., the highest dilution or minimum concentration of MAb that blocks viral replication). Nonneutralizing or neutralizing MAbs can be used in other serological assays such as enzyme linked immunosorbent assay, direct fluorescent assay, or indirect fluorescent

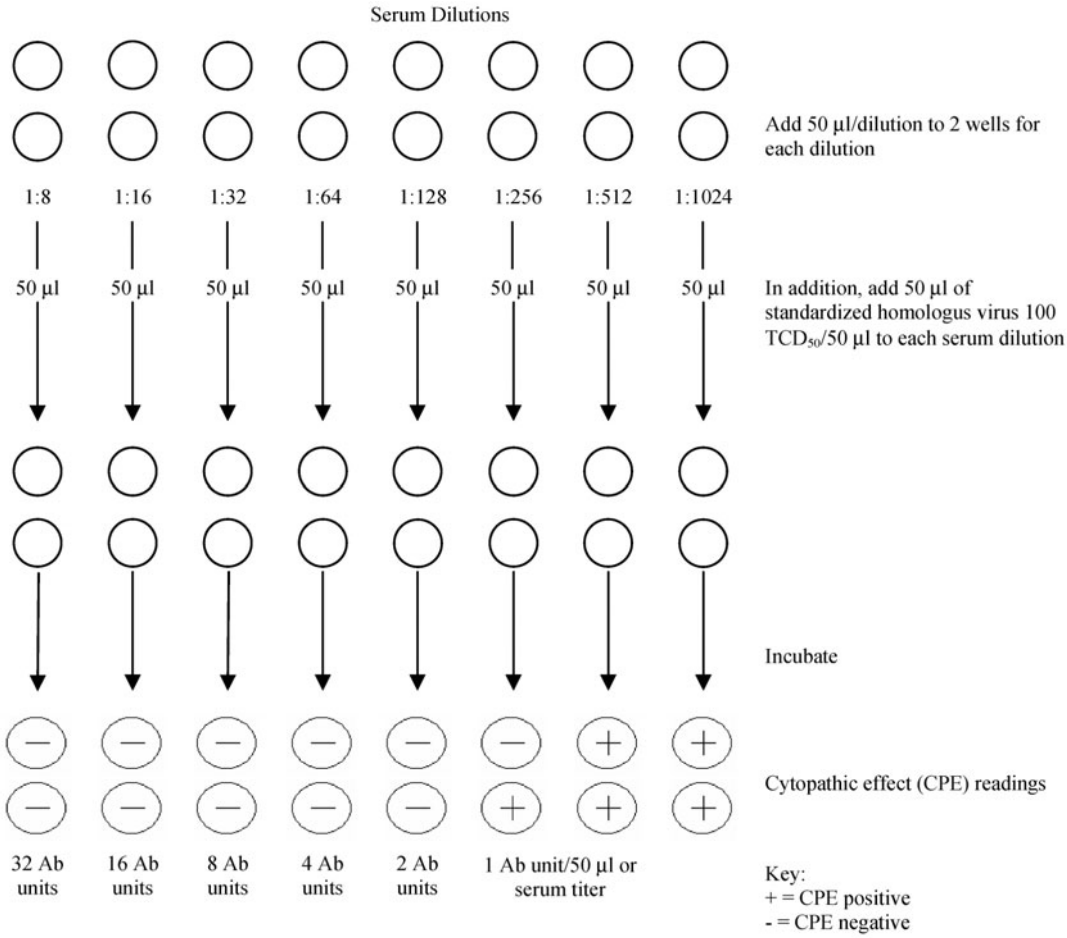

FIGURE 2 This serum titration demonstrates that the antibody titer is the highest dilution of antiserum that protects against the standardized virus.

assay. Neutralization is a specialized property that extends the range of application of a given MAb.

Earlier studies have shown that the neutralization titer is reduced in certain circumstances by heat, repeated freezing-thawing, and prolonged storage. The neutralization titer may be restored by adding "accessory factors," complement or other undefined substances that are present in freshly collected serum or serum that has been maintained in a frozen state to the virus-serum mixtures. These factors may also be eliminated by heating the sera to be tested at 56°C for 30 min prior to testing.

Assessing Serum Neutralizing Titer and Ab Units

Serum titration for neutralizing antibodies:

1. Prepare serial twofold dilutions of the serum.
2. Mix each dilution with an equal volume of standardized virus (usually 100 $TCID_{50}$ or its equivalent).
3. The virus and serum mixtures are incubated for 1 hr at room temperature or at 37°C. The time and temperature for incubating the virus and serum mixtures vary with different viruses and protocols.
4. Inoculate a susceptible host system with each virus-serum mixture or add a predetermined number of freshly trypsinized cells to each well.
5. Incubate until the virus titer reaches 100 $TCID_{50}$ or for a set number of days.
6. Read viral titration and calculate viral titer and score for growth of virus in virus-serum wells.

Interpretation

The serum antibody titer is the reciprocal of the highest dilution of antiserum protecting against the virus. The endpoint dilution contains 1 Ab unit per unit of volume (Fig. 2). The standardized concentration generally used in the neutralization test for identifying a virus is 20 Ab units/volume.

Host System

Neutralization tests require a living host system to monitor for virus growth. Following incubation of the virus-serum mixture, the susceptible host system is inoculated. The type of host used is primarily determined by the infectious and lethal capacity of the virus, as well as host availability.

Cell Cultures

Whenever possible, cell cultures are the preferred host system for performing the neutralization assays because they are susceptible to a wide range of viruses, are readily available, and have no immune system to influence the test. The cells

may be a preformed monolayer or a suspension of a predetermined number of freshly trypsinized and counted cells mixed with virus serum.

Animal Hosts

Neutralization tests using animal hosts are performed infrequently today. When needed, mice are most frequently used. The age of the mouse has a great influence on its susceptibility to disease; suckling and newborn mice are generally more susceptible. Typically, death, paralysis, or other symptoms are used to identify unneutralized virus. Assays with death as an outcome are reported as lethal dose (50% lethal dose) while assays with an infectious endpoint are reported as infectious dose (50% infectious dose).

Controls

For a valid test, cells in the uninoculated control wells must remain healthy and show no degeneration that could result from nonspecific cell destruction. Nonspecific effects due to aging, pH, medium, or other toxic effects may alter the results. The virus must achieve a minimum titer; while 100 $TCID_{50}$ is the usual target titer, a range (e.g., from 32 to 320 $TCID_{50}$) may be acceptable.

TEST PROCEDURES

Constant Virus, Varying Serum

This method is used to diagnose viral infection by testing paired host sera for a significant rise in the neutralizing antibody titer against a challenge virus. The assay requires both the virus that is being tested and paired sera (i.e., collection of an acute-phase serum taken as early in the course of infection as possible and a convalescent-phase serum collected 2 to 3 weeks after the onset of symptoms). The procedure is as follows:

1. Prepare serial twofold dilutions of acute- and convalescent-phase sera (dilutions may vary depending on a number of considerations).
2. Titrate virus to contain 1, 10, 100, 1,000, and 10,000 $TCID_{50}$.
3. Add well standardized virus diluted to contain a known dose (usually 100 $TCID_{50}$ or equivalent) to serum dilutions.
4. Incubate the virus-serum mixture for 1 h at the appropriate temperature.
5. Inoculate a susceptible host system or add a fixed number of freshly trypsinized cells.
6. Assay for viral growth and compute virus titer and serum neutralizing titers.

Interpretation

The highest dilution of serum protecting the host against the virus is the serum titer. The cell controls must remain healthy and the virus titer must fall within a predetermined value; a preselected titer of between 32 and 320 $TCID_{50}$ may be considered acceptable for a valid test. CPE or any of the previously mentioned methods for determining viral titer may be used. A fourfold rise in antibody titer between acute- and convalescent-phase sera is usually considered diagnostically significant (Fig. 3). Nonneutralized virus is recognized by the growth of virus in wells inoculated with virus serum mixtures.

Constant Antiserum, Varying Virus

This format is used for identification of a virus. One or more dilutions of virus selected to yield an infectious dose in the range of 32 to 320 $TCID_{50}$ are mixed with a single antiserum dilution selected to contain ≥20 Ab units. (The antiserum was previously standardized for specificity and titer to contain 20 Ab units of sera per volume and to have no or minimum cross-neutralization with related viruses.) The procedure is as follows:

1. Add 20 Ab units of specific antiserum to six wells.
2. Dilute virus to contain 1, 10, 100, and 1,000 $TCID_{50}$.
3. Add 10, 100, and 1,000 $TCID_{50}$ to two wells each with specific immune serum.
4. Incubate for 1 h.
5. Add predetermined number of freshly trypsinized cells to all wells or transfer to plates containing preformed cell monolayers.
6. Observe daily until 100 $TCID_{50}$ are present in the virus titration or until a set number of days are complete.
7. Read, record results, and calculate endpoints for viral titration and neutralization.

Interpretation

The virus is identified if at least 32 $TCID_{50}$ are neutralized by 20 Ab units and the cell controls remain healthy. CPE or any of the previously mentioned methods for determining viral titer may be used. Nonneutralized virus is recognized by the growth of virus in wells inoculated with virus-serum mixtures.

Constant Virus, Constant Antiserum

This method is similar to the "constant virus, varying antiserum" method, except that only a single viral dose is used in the virus-antibody mix. The "constant virus, constant antiserum" method is also used to identify a virus or confirm results obtained from other methods, (e.g., typing of virus with multiple serotypes section using pools of immune sera). The procedure is as follows:

1. Add ≥20 Ab units of specific antiserum to 2 wells.
2. Dilute virus to contain 1, 10, 100, 1,000, and 10,000 $TCID_{50}$ and add to wells in plate.
3. Add virus dilution estimated to contain 100 $TCID_{50}$ to two wells with immune serum.
4. Incubate for 1 h.
5. Transfer to monolayer or add predetermined number of freshly trypsinized cells to all wells.
6. Observe daily until 100 $TCID_{50}$ are present in the virus titration or until a set number of days are complete.
7. Record results and calculate endpoints.

Interpretation

The virus isolate is identified if the virus titers to at least 32 $TCID_{50}$ and there is no evidence of viral growth (CPE) in the cell cultures inoculated with the virus-serum mix. The presence of antibody in serum also can be determined by running a single dilution of a test serum against a standard dose of known virus. Neutralization indicates the presence of specific antibody.

Typing of Viruses with Multiple Serotypes

Serotyping of closely related viruses with multiple serotypes (e.g., adenoviruses, enteroviruses, and rhinoviruses) is

Test I

Serum sample	Dilution									Controls
	Undiluted	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	
Acute phase	+	+	+	+	+	+	+	+	+	–
Convalescent phase	+	+	+	+	+	+	+	+	+	–

The results of this test indicate that the individual has not been exposed to the tested virus.

Test 2

Serum sample	Dilution									Controls
	Undiluted	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	
Acute phase	–	–	+	+	+	+	+	+	+	–
Convalescent phase	–	–	–	–	–	+	+	+	+	–

The results of this test demonstrate an eightfold rise in antibody titer between acute- and convalescent-phase sera, suggesting recent infection with the tested virus.

Test 3

Serum sample	Dilution									Controls
	Undiluted	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	
Single serum	–	–	–	–	–	–	+	+	+	–

The antibody titer of 160 for a single serum sample indicates past infection with the tested virus. A high antibody titer is no guarantee of recent infection.

Test 4

	Dilution									Controls
	Undiluted	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	
Acute phase	–	–	–	–	–	+	+	+	+	–
Convalescent phase	–	–	–	–	–	+	+	+	+	–

The antibody titers of 80 for the paired sera indicate infection with the tested virus at some time in the past. It is very important to collect the sera at the proper time: if the acute-phase serum is drawn too late, the antibody titer may have already risen to the point that, when compared with the convalescent-phase serum titer, a rise in antibody may not be apparent.

FIGURE 3 Examples of serologic test results: +, virus infectivity; –, no virus infectivity.

challenging. Traditionally, these viruses have been recognized by their pattern of growth in cell culture; the specific serotype is defined by the absence of cross-neutralization between serotypes and is identified by use of type-specific antibody. These methods can be labor-intensive and costly. For example, human enteroviruses, of which there are close to 100 serotypes, are identified in cell culture based on the CPE, growth characteristics, and ultimately, neutralization by type-specific antiserum. MAbs for typing 18 enteroviruses are commercially available. These MAbs can be used individually to identify a specific serotype or as pools to identify a grouping of human enteroviruses. Human adenoviruses growing in cell culture are identified by their reaction with adenovirus group-reactive MAb. However, with 52 recognized serotypes of adenovirus, routine serotyping is prohibitive with regard to access to specific neutralizing serum, labor, and cost. Rhinoviruses are recognized by CPE and growth characteristics but may be difficult to differentiate from enteroviruses. The acid lability test is the definitive test for differentiating enterovirus from rhinovirus, but it is cumbersome and rarely used. Typing of rhinoviruses, with more than 100 human serotypes, is particularly challenging.

Molecular methods for determining serotype have been developed for the enteroviruses (Oberste et al., 1999) and adenoviruses (Lu and Erdman, 2006); however, they are not widely available. Additionally, the use of molecular methods for serotyping has limitations. Because serotype was established according to functional features of a virus (e.g., neutralization by a type-specific antiserum), molecular typing may not always predict phenotype. For example, prime

Composition of serum pools

Serum pool numbers	7	8	9	10	11	12
1	AV 1	AV 2	AV 3	AV 4	AV 5	AV 6
2	AV 7	AV 8	AV 9	AV 10	AV 11	AV 12
3	AV 13	AV 14	AV 15	AV 16	AV 17	AV 18
4	AV 19	AV 20	AV 21	AV 22	AV 23	AV 24
5	AV 25	AV 26	AV 27	AV 28	AV 29	AV 30
6	AV 31	AV 32	AV 33	AV 34	AV 35	

Identification of Isolates

		Neutralization				
	Serum pool numbers	7	8	9	10	11	12
	1						
	2						
Neutralization	3		X				
	4						
	5						
	6						

Identified as Adenovirus type 14

FIGURE 4 Intersecting pool scheme for identification of adenoviruses.

strains are not likely to be recognized by sequence analysis. Prime strains are viruses that have changed over time so that they are no longer neutralized by antiserum to the prototype virus (the original virus used to establish that serotype). For example, antiserum made to prototype echovirus 11 will neutralize echovirus 11 viruses but not echovirus 11 prime. However, antiserum made to echovirus 11 prime will neutralize the prime strain and the prototype virus. Persons immune to echovirus 11 prototype virus may be susceptible to the prime strain. This antigenic change can be identified by neutralization assay but not by sequencing. In the future, if the serotype is defined exclusively by sequence, the term may lose its biological significance.

Serotyping by Intersecting Pools of Hyperimmune Antisera

Serologic methods using serum pools are available to simultaneously identify multiple closely related serotypes in a single assay (Lim and Benyesh-Melnick, 1960; Schmidt et al., 1961). For example, the Lim and Benyesh-Melnick pools for enteroviruses use 15 pools in two sets of eight and seven. The first eight identify 42 echoviruses, coxsackie B viruses, and some coxsackie A viruses. The second set of seven pools identify 19 coxsackie A viruses. This method simplifies serotyping but is subject to misidentifications because it depends on neutralization in one, two, or three pools without any internal control. The results of the pools should always be confirmed by a "constant virus, constant antiserum" assay using type-specific antiserum. The intersecting pool scheme is designed to reduce the probability of misidentification; to identify a virus, neutralization must occur in two pools, and the serotype is read off an intersecting chart (Fig. 4).

At the California Department of Public Health Viral and Rickettsial Disease Laboratory (VRDL), procedures using intersecting immune serum pools have been developed and standardized for serotyping enteroviruses, 89 types of rhinoviruses, and 35 adenoviruses.

In the following paragraphs, detailed protocols for serotyping adenovirus, a group including 52 serotypes, are described. These protocols have a number of steps, including first testing by a neutralization assay using antiserum to the seven most frequently identified serotypes, adenoviruses 1 to 5, 7, and 21 (Schmitz et al., 1983), followed by a second neutralization assay using intersecting pools that identify 35 adenoviruses. Using this protocol, adenovirus serotypes from 36 to 52 are identified by sequencing and type-specific neutralization.

Neutralization Assay for the Seven Most Frequently Identified Serotypes of Adenovirus (Malasig et al., 2001)

1. Load one well per isolate of a 96-well plate with 20 Ab units of specific antiserum for each of the seven serotypes (adenoviruses 1 to 5, 7, and 21).
2. Titer the virus from undiluted to 10^{-5}.
3. Mix a 10^{-2} dilution of the isolate with each aliquot of antiserum.
4. Incubate the dilutions of the virus titration and the virus-antiserum mix for 1 h.
5. Add 1×10^5 A-549 cells in 100 µl to each well.
6. Incubate the plates until the viral titration reaches 100 $TCID_{50}$ or for a set period of time.
7. Determine the endpoints by observing for CPE or the 50% endpoint determined by absorbance following staining with neutral red dye for viable cells.

Interpretation

The virus is identified if at least 32 $TCID_{50}$ or the equivalent is neutralized by one of the immune sera. This assay is highly sensitive, specific, and efficient. If initial testing is negative for these seven serotypes, testing should proceed with the neutralization assay using intersecting pools.

Preparation of Intersecting Immune Serum Pools

1. Determine the titer of each serum; this endpoint dilution or antibody titer is the last dilution of serum demonstrating complete neutralization (no virus growth) and contains 1 Ab unit.
2. Twenty Ab units of each serum are required in the pool. To calculate 20 Ab units, divide the denominator of the dilution containing 1 Ab unit by 20, e.g., 320/20 = 16; thus, a 1/16 serum dilution contains 20 Ab units.
3. With the sera titered and the dilution containing 20 Ab units of serum calculated, incorporate the sera into the pool at the dilution that will give 20 Ab units.
4. Prepare the pools (Fig. 3).
5. Standardize by testing the pools against 100 $TCID_{50}$ each of all prototype adenoviruses.

   The titers of the sera must be high enough so that their combination in a pool will result in a total serum concentration not greater than 10%. These immune serum pools are now ready for viral serotyping.

Intersecting Pools to Determine 35 Adenovirus Serotypes

1. Add an aliquot from each pool to 1 well for each isolate to be typed.
2. Titrate virus from undiluted to 10^{-5}.
3. Mix the 10^{-2} dilution of the virus isolate with each pool.
4. Incubate for 1 h.
5. Add a predetermined number of freshly trypsinized cells to each well or transfer the mixture of pooled antiserum and virus to a plate with a preformed monolayer.
6. Incubate at 36°C in 5% CO_2.
7. Score for viral growth using an appropriate assay.

Interpretation

The test is satisfactory if at least 32 $TCID_{50}$ of the virus isolate was mixed with each serum pool and neutralization occurs in two wells so that reading off of the grid (Fig. 4) allows the isolate to be typed.

In the event that the isolate is not one of these 35 serotypes, sequence analysis of a portion of the hexon gene can be used to determine the serotype (Lu and Erdman, 2006). For all results determined by either intersecting pools or sequencing, the serotype should be confirmed by type-specific neutralization using the "constant virus, constant antiserum" assay.

SUMMARY

The neutralization test remains relevant today. It is used for typing viruses and for diagnosing infection based on the host's immune response. It is also a research tool for dissecting how antibodies protect the host from infection and probing viral function. Although the test is currently used less than it was in the past, it will retain an important role in diagnostic virology and research.

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Hemadsorption and Hemagglutination Inhibition

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10

HEMADSORPTION

Two genera of common respiratory viruses, the influenza and parainfluenza viruses, and several other viruses, mumps virus and Newcastle disease virus, replicate in cell cultures but frequently do not produce cytopathic effects. The cell lines routinely used for isolation of these viruses include primary monkey kidney cells and continuous cultures of Madin-Darby canine kidney cells and rhesus monkey kidney (LLC-MK2) cells. Although the replication of these viruses may not produce cytopathic effects, the presence of replicating virus can be detected by hemadsorption (Hsiung, 1982; Swenson, 1992a: Leland, 1996).

The final step in the maturation phase of these viruses is the budding of the virion through the plasma membrane of the cell. The insertion of viral proteins necessary for budding of virus from the cell membrane facilitates the binding of red blood cells (RBCs) to the infected cells. This process is referred to as hemadsorption, and the RBCs from several species (human O, chicken, or guinea pig) can be used in this method. This process is relatively simple. A suspension of RBCs is added to the cell monolayer, and the mixture is incubated at the required temperature and then observed microscopically for adsorption of RBCs to the cells in the monolayer.

Materials

Guinea pig RBCs

A 10% (vol/vol) suspension of RBCs in Alsever's solution is used. This suspension is stable for 1 week when stored at 4°C.

Uninoculated cell culture controls

Cell cultures inoculated with patient sample(s)

Cell cultures inoculated with known hemadsorbing strains of virus

Method

1. Transfer 5 ml of the RBCs in Alsever's solution to a graduated centrifuge tube, add 5 ml of phosphate-buffered saline (PBS), mix well, and centrifuge at 900 × *g* for 5 min.

2. Discard the supernatant, and resuspend the RBC pellet in 10 ml of PBS.

3. Repeat the pelleting and resuspension steps two more times.

4. After discarding the supernatant from the final centrifugation, determine the packed cell volume of the RBCs.

5. Resuspend the RBCs in PBS to a final concentration of 10% (vol/vol). The stock suspension of RBCs should not be stored for more than 1 week at 4°C.

6. On each day of testing, prepare a 0.4% (vol/vol) suspension of guinea pig RBCs in PBS from the 10% (vol/vol) stock solution of guinea pig RBCs.

7. Remove the cell culture fluid from each monolayer in a biological safety cabinet. The cell culture fluid can be saved in a sterile tube or discarded depending on the method used to identify hemadsorbing virus.

8. Add 0.2 ml of the 0.4% RBC solution to each cell culture. The guinea pig RBC suspension should cover the monolayer but not reach beyond the neck of the tube.

9. If mumps virus is in the healthcare provider's request, incubate the cell cultures at 4°C for 30 min.

10. Rock each tube gently, and then observe each monolayer microscopically. The uninoculated cell monolayer generally does not have cells displaying hemadsorption (Fig. 1A). However, as the cells in the monolayer age, they become sticky and can appear to have RBCs that are hemadsorbing. The cell cultures inoculated with a known hemadsorbing virus should have RBCs adsorbed to the cells (Fig.1B). The monolayers can show varying degrees of hemadsorption, and at times, the supernatant will also have hemagglutination of the RBCs.

11. If other hemadsorbing viruses are included in the test request, the laboratory will perform a second hemadsorption by incubating the tubes at room temperature for 30 min and observing them as described above. The room temperature incubation and observation may be the only hemadsorption assay performed if mumps virus isolation is not requested.

12. For hemadsorption-negative cultures, the RBC suspension can be removed, fresh cell culture media can be added, and the cultures can be reincubated.

13. The identification of the hemadsorbing virus can be done with either the supernatant or the infected cells. The monolayer can be removed from the surface of the tube, and the cells are then attached to a glass slide and stained in either a direct or indirect immunofluorescent assay. The binding of virus-specific antibody is detected by direct observation of the stained cells with a fluorescent microscope. The supernatant can be tested by hemagglutination inhibition (HAI).

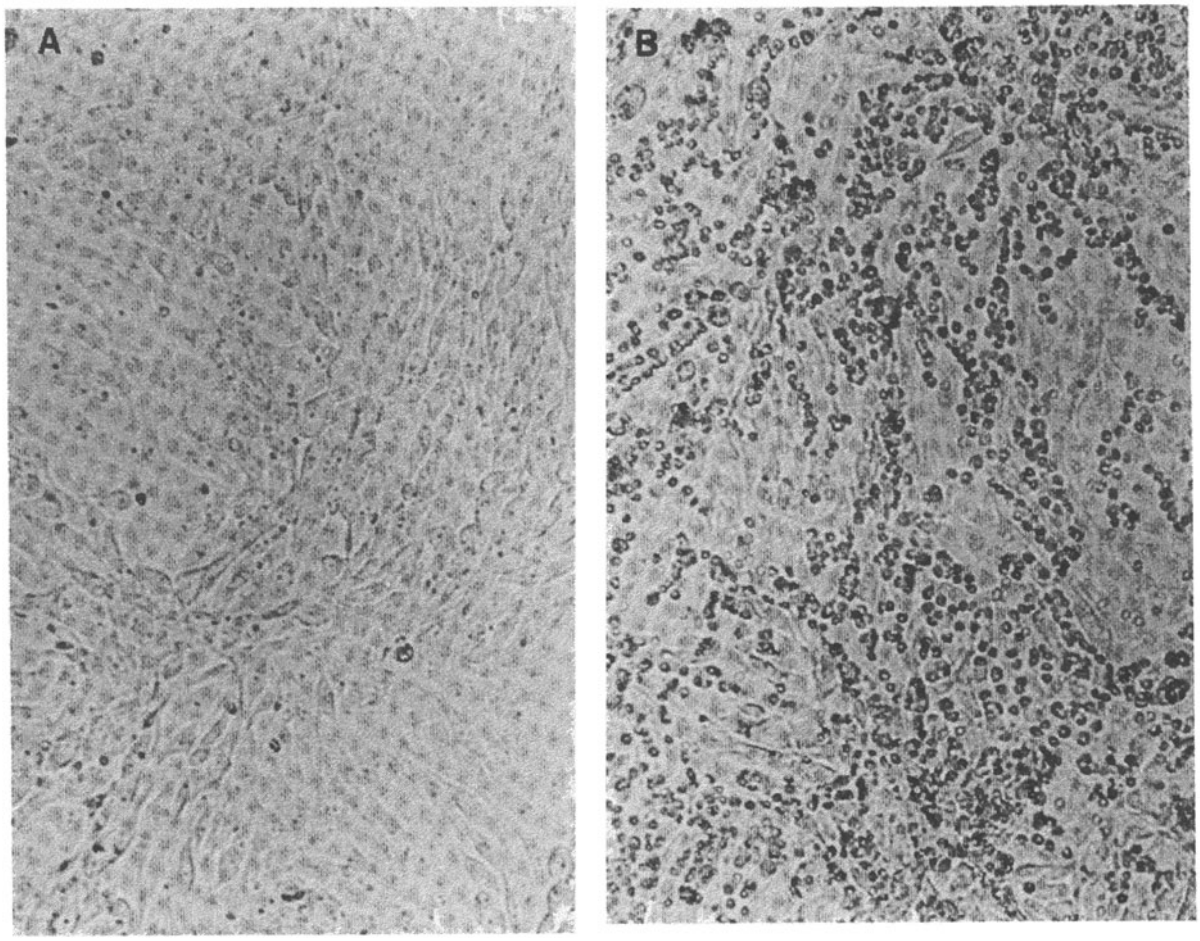

FIGURE 1 Hemadsorption. (A) Uninoculated primary monkey kidney cells; (B) primary monkey kidney cells infected with influenza A virus.

HAI TEST

A wide variety of viruses have the ability to bind with and then agglutinate RBCs (hemagglutination). Adenoviruses, arboviruses, some enteroviruses, influenza virus, parainfluenza viruses, mumps virus, measles virus, and reoviruses have this property (Hsiung, 1982; Schmidt and Emmons, 1989; Swenson, 1992b; Hodinka, 1999). The hemagglutination test can be used to detect either virus or the amount of hemagglutinin (HA) in a sample. Antibodies that have the ability to bind with either the virus or HA will prevent hemagglutination. This is the principle of the HAI test. In the past, the HAI test was used routinely in clinical virology laboratories to detect antibody to several of these viruses. However, the HAI test is no longer a routine procedure in most clinical laboratories for either serologic diagnosis or virus identification. The movement away from the HAI test is primarily due to the time required to perform the test, even though it is relatively easy to perform and requires only inexpensive equipment and reagents (Mahony and Chernesky, 1999).

Serological diagnosis by the HAI test is accomplished by making serial dilutions of a patient's serum, mixing each dilution with a fixed amount of viral HA, and then adding an RBC suspension. The animal species from which the indicator RBCs are collected is dependent on the viral HA used in the assay. If the serum contains antibody, the virus will be bound and unable to agglutinate the RBCs. If there are no antibodies in the patient's serum, the virus will agglutinate the RBCs. The reciprocal of the highest dilution of the patient's serum that will completely inhibit agglutination is termed the antibody titer (Hsiung, 1982; Schmidt and Emmons, 1989; Swenson, 1992b).

Serological diagnosis by the HAI test can be complicated by the presence of nonspecific viral inhibitors (nonantibody) and red cell agglutinins in the patient's sera (Hssiung, 1982; Swenson, 1992b; Hodinka, 1999). The presence of such inhibitors can give rise to false-positive results in the HAI test. Therefore, several procedures were developed to remove the inhibitors or RBC agglutinins. These procedures included RBC adsorption, kaolin adsorption, receptor-destroying enzyme treatment, and heat inactivation or potassium periodate treatment. The detailed procedures for serum pretreatment have been described elsewhere (Hsiung, 1982; Swenson, 1992b).

The most common use of the HAI test in laboratories today is for subtyping of influenza virus isolates by state health department- or World Health Organization-collaborating influenza surveillance laboratories. A detailed procedure for the typing of influenza virus isolates is presented by Swenson (1992b).

The specificity of the HAI test varies with the virus. The reaction can be highly specific for certain viruses (influenza-parainfluenza groups) and less specific for other viruses (arboviruses). A procedure for measuring antibody to influenza viruses is presented below.

Materials

Blood from a guinea pig or other mammalian or avian species in Alsever's solution at a 10% (vol/vol) concentration (This suspension is stable for 2 weeks when stored at 4°C.)

Amniotic, allantoic, or cell culture fluid containing influenza virus

Alsever's solution

PBS, 0.01 M, pH 7.2

Disposable microtiter plates, "U" type

Calibrated diluting loops, 0.025 and 0.050 ml

Dropping pipettes, 0.025 and 0.050 ml per drop

Blotter paper with calibrating circles

Microtiter plate reading mirror

37°C and 56°C water baths

Receptor-destroying enzyme, 100 U/ml

Preparation of RBC Suspension

To prepare the RBC suspension, see steps 1 through 5 of the hemadsorption method, above.

Titration of HA

1. A 0.5% (vol/vol) solution of RBCs in PBS is prepared from the stock suspension (1 ml of 10% [vol/vol] RBCs added to 19 ml of PBS).
2. Prepare stocks of the influenza A and B viruses currently circulating in one of the following formats: cell culture fluid, allantoic fluid, or amniotic fluid.
3. Dilute the influenza A and B stock solution 1:10 in PBS and add 0.1 ml to the first well of each row on the microtiter plate. Add 50 µl of PBS to wells 2 through 9 in that row.
4. Place a calibrated 0.05-ml diluter in well 1 and transfer the contents to well 2.
5. Move the diluter from well to well, resulting in a dilution scheme from 1:10 to 1:2560.
6. Prepare an RBC control by adding 0.05 ml PBS to a well on the microtiter plate.
7. Add 50 µl of the 0.5% (vol/vol) RBC suspension to each well using the calibrated dropping pipettes.
8. Mix the contents of the plate gently, cover the plate, and incubate it at room temperature until the RBC controls form a tight button (1 to 2 h).
9. The HA titer is the highest dilution of virus capable of causing agglutination; agglutinated RBCs form a lattice on the bottom of the well, while nonagglutinated RBCs will form a discrete button at the bottom of the well (Fig. 2). The HA titer is the reciprocal of the highest dilution of virus showing agglutination and represents 1 HA unit/0.05 ml of virus. Dilute the virus suspension to contain 4 HA units/0.025 ml (or 8 HA units/0.05 ml) for the HAI test. If the HA titer is 160, then the original virus stock will be diluted 1:20 for the HAI test.

HAI Test for Influenza Virus

1. Acute- and convalescent-phase sera, pretreated to remove nonspecific inhibitors, should be tested for antibodies to one or more of the circulating strains of influenza virus. The HA titer for each virus used in the assay should be 4 HA units/0.025 ml.

2. Reference antiserum for each virus should be included to confirm the identities of virus strains used in the test.

3. Prepare a 1:10 dilution of the pretreated acute- and convalescent-phase serum in PBS and add 0.05 ml of this dilution to well 1 of a dedicated row.

4. Add 0.025 ml of PBS to wells 2 through 9 in each row.

5. Prepare twofold dilutions of each treated serum using a calibrated 0.025 ml microtiter diluter. This is accomplished by placing the diluter in the first well of each row and sequentially transferring 0.025 ml from well 1 to well 9. The serum dilutions span the range from 1:10 to 1:2,560. This same dilution scheme would be used with the control antiserum.

6. A control reaction in which only the serum and cells are added is setup for each serum.

7. Add 0.025 ml of each virus suspension (4 HA units/0.025 ml) to each serum dilution and the serum control.

A back titration of the virus suspension is included in each HAI assay. Five wells in a row are used for each back titration. Fifty microliters of PBS is added to wells 1 through 5 and three additional wells. Fifty microliters of the virus suspension is added to well 1. Twofold dilutions of the virus suspension are prepared using a precalibrated 0.05-ml microtiter diluter. The diluter is placed in the first well, and then 0.05 ml is transferred sequentially from well 1 to 4.

8. Gently shake the microtiter plate after serum dilutions and HA have been added, cover the plate, and incubate at room temperature for 30 min.

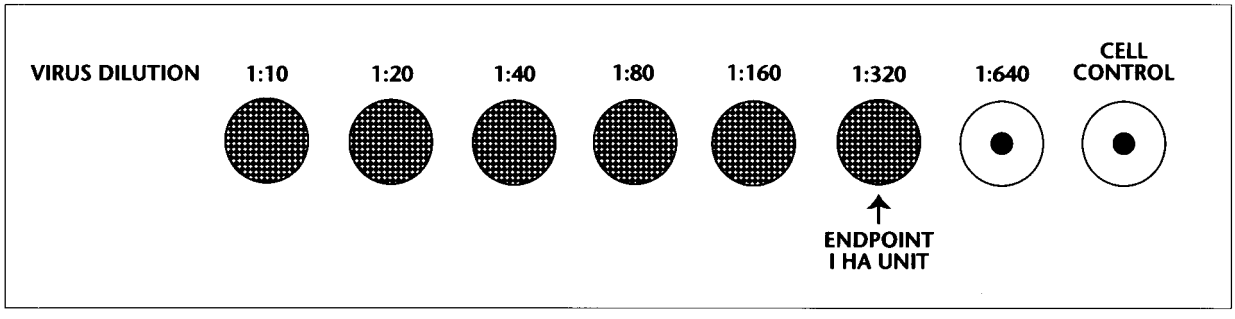

FIGURE 2 HA endpoint determination.

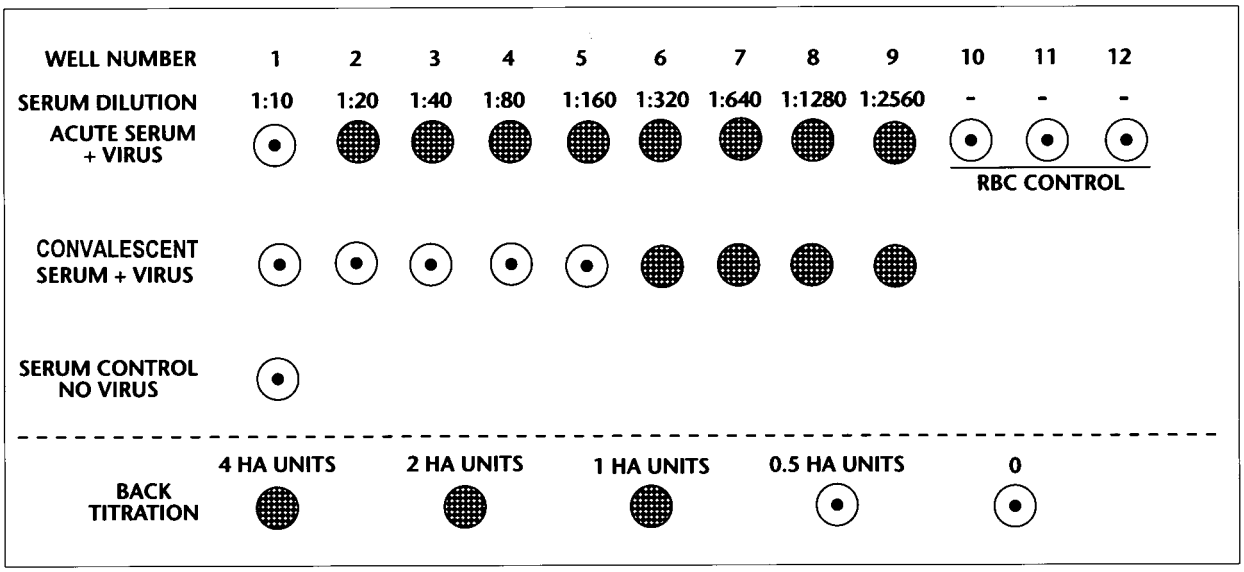

FIGURE 3 Determination of HAI titers for acute- and convalescent-phase sera.

9. Add 0.05 ml of the 0.5% (vol/vol) RBC suspension to each well using calibrated dropping pipette. This suspension should be added to all the serum dilutions, the back titration, serum controls, and RBC control wells.

10. Gently shake the microtiter plate and incubate the plate at room temperature until the RBC control has a button at the bottom of the well (30 to 60 min).

11. The back titration of the virus suspension should show hemagglutination in the first two to three wells, indicating the dilution of the virus suspension was correct. The serum and RBC controls should show the absence of agglutination.

12. The HAI titer of each serum is defined as the highest dilution of serum that completely inhibits hemagglutination. The HAI titer of the acute-phase serum is 10, and for the convalescent serum the titer is 160. A fourfold or greater rise in HAI titers is interpreted as significant and is indicative of recent influenza virus infection or vaccination (Hsiung, 1982; Swenson, 1992b). An example of an acute- and convalescent-phase serum titrated against a single influenza virus strain is presented in Fig. 3.

Other Viruses to Which the HAI Test Is Applicable

The procedure described above is applicable to the parainfluenza viruses, rubella virus, measles virus, reovirus, adenovirus, and togavirus groups with some modifications. The appropriate chapters in this text should be consulted for details. Modifications that are necessary for these viruses are summarized below (Hsiung, 1982; Swenson, 1992b).

1. Modifications for parainfluenza viruses include the use of human O, guinea pig, or chicken RBCs, pretreatment of the serum with receptor-destroying enzyme heat treatment, and in a majority of cases, adsorption with guinea pig RBCs.

2. Modifications for rubella virus include the following: use 1-day-old chick or goose RBCs, the diluent is HEPES-saline-albumin-gelatin, and sera must be treated with $MnCl_2$-heparin and adsorbed with chick RBCs to remove nonspecific inhibitors and agglutinins.

3. Modifications for the adenovirus group include the following: rhesus monkey RBCs are used for group 1 adenovirus serotypes, and rat RBCs are used for group 2 adenovirus serotypes. The sera must be heat inactivated at 56°C for 30 min and adsorbed with the type of RBCs used in the HAI test.

4. Modifications for the reoviruses include the use of human O RBCs and pretreatment of the sera by both heat inactivation and kaolin adsorption.

5. Modifications for members of the arbovirus group include use of buffers with pH values ranging from pH 6.0 to 7.4 and pretreatment of the sera with heat inactivation and kaolin adsorption.

6. Modifications for the serotypes of enteroviruses that are capable of HAI include using human O RBCs, changing the incubation temperature dependent upon the serotype, and pretreatment of the sera by heat inactivation and kaolin adsorption. Coxsackie viruses A-20, A-21, and A-24, and echovirus types 3, 11, 13, and 19 agglutinate at 4°C. Coxsackie viruses B-1, B-3, and B-5 and echovirus types 6, 7, 12, 20, and 21 hemagglutinate at 37°C.

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Immunoglobulin M Determinations

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11

The presence of specific antibody activity due to immunoglobulins (Igs) in serum was reported as early as the 1930s (Heidelberger and Pederson, 1937). Subsequent studies demonstrated that the first Igs to appear after a primary antigenic stimulus were of the IgM class. These IgM antibodies reportedly disappeared rapidly, usually within a few weeks, and were replaced by IgG antibodies that persisted for a longer period, by a process now known as Ig-class switch recombination (Geha and Rosen, 1994). Decades later, Schluederberg (1965) suggested for the first time that detection of virus-specific IgM antibodies could be of value in recognizing recent virus infections. Today, IgM determinations have become routine procedures used in many diagnostic virology laboratories.

The transient nature of the IgM antibody response appears to hold true for most primary virus infections; virus-specific IgM antibodies typically appear 7 to 10 days after primary infection, reach maximal levels within 2 to 3 weeks, and then decline to undetectable levels after about 3 months. In contrast, IgG antibodies typically persist for years, if not indefinitely (Fig. 1). Consequently, detection of virus-specific IgM antibodies in a single serum specimen is now well established as a potentially valuable method for the rapid diagnosis of recent or current virus infections. Such an approach provides a considerable advantage over classical serological testing, which requires the demonstration of a significant rise in antibody titer between acute- and convalescent-phase serum specimens. For this approach to be successful, however, the IgM antibody response must be virus specific, transient (i.e., present only with recent infection by the particular virus), and measurable with adequate reliability and sensitivity.

METHODS USED FOR IgM ANTIBODY DETERMINATION

Since the introduction of the first applications of IgM determination in diagnostic virology, a variety of methods have been developed and applied for this purpose (for a more complete review, see Meurman, 1983). These methods can generally be separated into three groups (Table 1): (i) those based on comparing IgM titers before and after chemical inactivation of serum IgM, (ii) those based on the physicochemical separation of IgM from other serum Ig classes, and (iii) those based on solid-phase immunologic detection of IgM antibodies. This chapter will discuss the relative merits of each of these approaches.

Methods Based on Chemical Inactivation of IgM

One of the earliest methods for determination of virus-specific IgM antibodies involved pretreatment of serum with mercaptans, such as 2-mercaptoethanol and dithiothreitol, that have the capacity to selectively split IgM molecules into immunologically inactive forms by breaking the disulfide bonds between the polypeptide chains (Banatvala et al., 1967). A fourfold or greater decrease in antibody titer following mercaptan treatment was considered indicative of the presence of virus-specific IgM antibodies. To be effective, however, at least 75% of the total virus-specific antibody must be of the IgM class. Because this is the case only during the very early stages of most virus infections, the diagnostic value of this approach is quite limited. Furthermore, pretreatment with mercaptans can give variable results; with insufficient treatment, IgM monomers can reassociate and regain immune reactivity, resulting in false-negative reactions, and too rigorous treatment can reduce IgG molecules, producing a false-positive result. For these reasons, this method is no longer acceptable for detection of virus-specific IgM, but can be used to confirm successful separation of IgM antibodies by other methods.

Methods Based on Physicochemical Separation of IgM

Physicochemical separation methods were originally developed to separate IgM antibodies from other serum Igs to facilitate assay by conventional serological tests, e.g., complement fixation (CF) and hemagglutination inhibition (HI) assays. It was later recognized that IgM separation could also benefit some solid-phase immunoassays as well (see below).

Sucrose Density Gradient Centrifugation

One of the earliest methods for recovering IgM antibodies employs high-speed centrifugation of serum on sucrose gradients. Because IgM proteins have a higher sedimentation coefficient (19S) than other Igs (7 to 11S), IgM antibodies can be physically separated from other antibodies by rate-zonal centrifugation on sucrose gradients. Lipoprotein molecules, including most of the nonspecific inhibitors of rubella hemagglutination, have a low density and therefore remain near the top of the gradient following centrifugation.

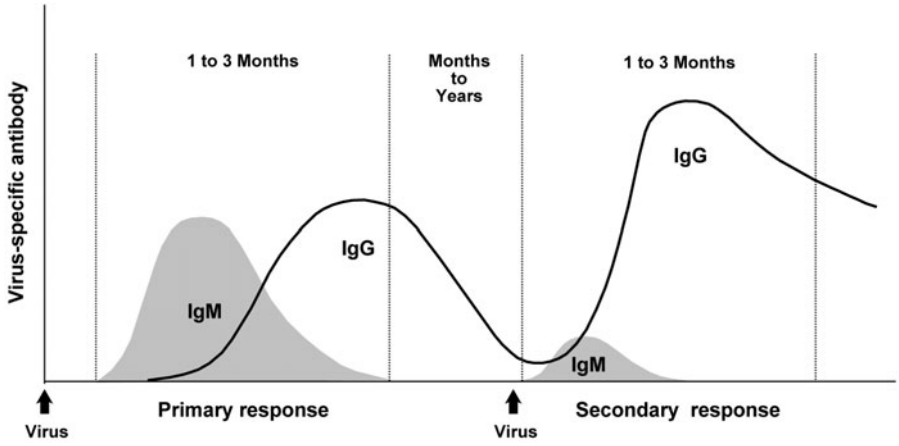

FIGURE 1 Primary and secondary immune responses to a hypothetical virus infection. After primary exposure to the viral antigen, there is usually an early and pronounced rise in IgM antibodies, followed by a gradual rise in IgG. IgM antibodies then typically decline to undetectable levels within 1 to 3 months, whereas IgG antibodies can persist for years. Because immunological memory develops, reexposure to the same or similar virus usually results in an early and enhanced rise in IgG antibodies and an attenuated IgM response.

This technique was introduced in 1968 for the rapid diagnosis of recent rubella virus infection by demonstrating the presence of IgM antibodies (Vesikari and Vaheri, 1968). Since that time, various modifications of the method have been published (Forghani et al., 1973; Caul et al., 1978) and the test has been applied to the diagnosis of many virus infections (Frösner et al., 1979; Al-Nakib, 1980; Hawkes et al., 1980).

To perform sucrose density gradient centrifugation, a density gradient is prepared by layering 1.4-ml amounts of 37%, 23%, and 10% (wt/vol) solutions of sucrose in 0.01 M phosphate-buffered saline, pH 7.2, on a 0.2-ml cushion of 50% sucrose in a 5-ml ultracentrifuge tube. The sucrose layers are allowed to equilibrate for 4 to 6 h at 4°C and then 0.2 ml of a 1:2 dilution of the test serum in phosphate-buffered saline (pretreated, if necessary, to remove nonspecific serum components that would interfere with the assay of IgM antibody) is carefully layered on top of the gradient. The gradient is then centrifuged at 157,000 × *g* for 16 h in a swinging-bucket rotor or for 2 h using vertical rotors with reorienting gradients (Frösner et al., 1979). Ten to 12 fractions of about 0.4 ml each are collected by puncturing the bottom of the tube. The IgM antibodies concentrate in the bottom three or four fractions, IgG antibodies separate primarily in fractions six to eight, and the lipoproteins (nonspecific inhibitors) remain near the top of the gradient. The first four fractions (i.e., those presumed to contain IgM) must be checked for both IgM antibody activity and presence of contaminating human IgG (Caul et al., 1974). The isolated IgM fractions can then be tested for antiviral activity by any suitable serological tests. Sucrose density gradient centrifugation combined with classical serological assays (e.g., CF and HI) are not as sensitive and are more cumbersome to perform than the more commonly used solid-phase IgM immunoassays (see below).

TABLE 1 Methods for IgM antibody determination

Method based on chemical inactivation of IgM
Alkylation reduction by mercaptans
Methods based on physicochemical separation of IgM
Sucrose density gradient centrifugation
Column chromatography
IgG absorption
Methods based on solid-phase immunologic detection of IgM
Indirect IgM immunoassays
Reverse, or capture, IgM immunoassays

Column Chromatography

Column chromatographic methods based on gel filtration (Frisch-Niggemeyer, 1982), ion- exchange chromatography (Johnson and Libby, 1980), and to a lesser extent, affinity chromatography (Barros and Lebon, 1975) have been used for many years to separate and isolate serum IgM antibodies. These methods offer simple and cost-effective alternatives to sucrose density gradient centrifugation while providing similar results if performed properly.

IgM separation on appropriately sized gels takes advantage of the differences in size between IgM molecules (molecular weight, 900,000) and other Igs (molecular weight, 150,000 to 400,000). Sephacryl S-300 or Sephadex G-200 columns (Amersham Biosciences, Piscataway, NJ) are two commercially available products that have been used for this purpose. Sephacryl S-300 is preferred because it does not need to be rehydrated and will allow high-flow-rate filtration of serum under pressure for more rapid specimen processing without over-packing or deforming the column (Morgan-Capner et al., 1980). Since serum lipoproteins and nonspecific cell agglutinins can elute from these columns along with the IgM, these substances must be removed prior to serum fractionation if they interfere with the assay. Serum, pretreated with heparin and $MnCl_2$ to remove lipoproteins and cell adsorbed to remove nonspecific agglutinins, is layered on the top of the gel column and eluted through the column with Tris-buffered saline (0.02 M Tris in 0.15 M NaCl, pH 7.5). Discrete fractions are collected for titration of antibody activity. Each new column should be standardized with known specific IgM-positive and IgM-negative serum samples. With both Sephacryl S-300 and Sephadex G-200 columns, IgM is

eluted in the first protein peak and IgG in the second; IgA may also be present in the first peak eluted from the Sephadex G-200 column but not from the Sephacryl S-300 column.

The specificity of gel separation of IgM for diagnosis of virus infections is very high, provided a number of factors that can cause false-positive results are taken into consideration (Pattison et al., 1976). Prolonged storage of serum at –20°C or bacterial contamination of the serum may make the pretreatment of it ineffective, resulting in false-positive results. Also, if the serum has been preheated at 56°C or higher, IgG may aggregate and therefore could elute into the IgM fractions after gel filtration. To minimize misinterpretation of the gel fractionation test, any presumptive IgM antibody activity in the first peak should be confirmed by an IgM-specific assay.

Ion-exchange chromatography, based on the differential binding of IgM and IgG antibodies to anion-exchange resins such as quaternary aminoethyl-Sephadex A50, also has been used for separation of IgM antibodies (Johnson and Libby, 1980). Serum loaded onto columns is washed, and most IgG and lipoproteins are eluted. IgM is recovered by elution at acid pH. As with sucrose density gradient fractionation and gel filtration, ion-exchange chromatography does not entirely eliminate IgG or IgA antibodies from the IgM fraction, and some loss of IgM antibodies can be expected (Elder and Smith, 1987; Elder et al., 1987).

IgG Absorption

A significant improvement in the speed and convenience of IgM separation was achieved with the development of methods for "absorption" of IgG antibodies. These methods are based on the capacity of certain bacterial cell wall proteins or their recombinant analogs to selectively bind and remove IgG in serum specimens. Briefly, the IgG absorbent is added to the serum specimen according to the manufacturer's instructions, incubated to allow binding between the absorbent and the IgG, and then centrifuged briefly to pellet the IgG-absorbent complex (some procedures do not require centrifugation). The supernatant is then ready for assay for IgM antibodies.

Ankerst et al. (1974) first applied this concept for detection of rubella IgM antibodies after absorption of serum with *Staphylococcus aureus* bacteria. Some strains of *S. aureus* possess cell wall protein A (SPA) that binds to the Fc receptor of IgG and can be used to absorb and remove the majority of the serum IgG. However, SPA binds strongly only to IgG subclasses IgG1, IgG2, and IgG4, but not IgG3 (Kronvall and Williams, 1969), which constitutes up to 5% of the original IgG antibody and has been shown to possess a disproportionately high level of antiviral activity (Beck, 1981). SPA can also absorb significant amounts of IgM with potential loss of assay sensitivity (Field et al., 1980) and is therefore no longer considered an acceptable method for IgM detection.

Streptococcal protein G (SPG), a cell wall protein from group G streptococci, offers improved characteristics over SPA for IgG absorption. SPG binds all four IgG subclasses and does not bind to IgM antibodies (Bjork and Kronvall, 1984). A recombinant form of SPG was successfully used to remove IgG antibodies in serum specimens, permitting detection of IgM and IgA antibodies to human immunodeficiency virus type 1 (Weiblen et al., 1990). In this study, the plasma specimens were incubated with a 50% suspension of SPG-agarose and centrifuged briefly, and the supernatant was recovered for assay. Up to a 99.95% reduction in IgG concentration was reported, although repeat treatments were necessary with specimens containing high-titer IgG. However, unlike SPA, SPG does not bind to IgA, IgD, or IgE antibodies, which also possibly could cause false-positive or false-negative results, depending on the method employed for IgM detection. To address this possibility, Fuccillo et al. (1992) pretreated serum with a combination of SPA and streptococci according to the method of Kronvall et al. (1979) to remove most serum IgG and IgA antibodies. For use with classical CF and HI assays, absorbed serum must be carefully evaluated to ensure that residual antibody activity is exclusively IgM.

IgG absorption methods are now routinely used to complement indirect solid-phase immunoassays for virus-specific IgM detection (see below). Preadsorption has been shown to reduce nonspecific IgM activity in serum (e.g., rheumatoid factors [RFs]) and to significantly increase the sensitivity of indirect IgM assays by removing most competing IgG. In addition to SPA and SPG, animal anti-human IgG antibody preparations have been used successfully to remove serum IgG antibodies. Martins et al. (1995) found anti-human IgG procedures to be the most effective and economical for IgM antibody testing. Commercial preparations of recombinant SPG (from, e.g., Pierce, Rockford, IL, and Invitrogen, Carlsbad, CA) and anti-human IgG (e.g., GullSorb [Meridian Bioscience, Cincinatti, OH] and RF removal reagent [Millipore, Temecula, CA]) are available separately or as part of some commercial antiviral IgM diagnostic kits.

Methods Based on Solid-Phase Immunologic Detection of IgM

The availability of class-specific antiglobulins, specifically, anti-human IgM, and solid-phase supports used to bind and separate immunoreactants, has revolutionized IgM detection and led to the rapid development of the wide range of assay formats currently used in diagnostic virology (Meurman, 1983). The major distinguishing features of solid-phase immunoassays are the choice of indicator label (e.g., fluorescein isothiocyanate, enzymes, radioisotopes, or erythrocytes) and solid phase (e.g., plastic 96-well microtiter plates, beads, tubes, or nitrocellulose/nylon membranes). Solid-phase immunoassays can be further differentiated into indirect and reverse, or "capture," forms, based on the orientation of the immunoreactants on the solid phase. The indirect and capture formats have advantages and disadvantages that will be described further below.

Indirect IgM Immunoassays

Indirect IgM immunoassays are characterized by the binding of viral antigen to a solid-phase surface followed by incubation with the serum specimen (Fig. 2). Specific IgM antibodies present in the specimen bind to the antigen and are subsequently detected with anti-human IgM antibody labeled with a suitable marker. Because of their technical simplicity and limited reagent requirements, indirect IgM immunoassays are particularly popular among virologists and commercial test kit vendors. However, two major problems often encountered with these assays can limit their sensitivity and specificity: (i) interference by IgM class RF and (ii) competition between specific IgG and IgM antibodies in patient serum specimens for available epitopes on the antigen bound to the solid phase. Each of these factors can play an important role in the reliability of an indirect IgM antibody assay system.

False-positive results can occur in indirect IgM assays when RF antibodies present in the serum attach to complexes of IgG antibody bound to the antigen on the solid phase (Fig. 3).

FIGURE 2 Schema of indirect solid-phase immunoassay for IgM antibody.

RF is antibody primarily of the IgM class that reacts with the Fc portion of bound IgG. RF is found in a high percentage of persons with rheumatoid arthritis and related connective tissue diseases and can also be found to varying degrees in patients with subacute bacterial endocarditis, chronic liver disease, parasitic infections, and tuberculosis, as well as during pregnancy and even among apparently normal healthy persons, particularly neonates and the elderly (Meurman, 1983; Fuccillo et al., 1992). Transient appearance of RF also has been associated with acute infection with parvovirus B19, measles, rubella, and cytomegalovirus and following heavy prophylactic vaccination. In practice, the effects of RF can vary with assays for different viruses without obvious explanation (Salonen et al., 1980).

Because indirect IgM immunoassays fix antigen directly to the solid phase, components of the cell line in which the virus was grown or expressed (i.e., cellular proteins and nucleic acids) may be present on the solid-phase surface and serve as targets for serum antibodies with specificity for these components, e.g., antinuclear antibodies that cross-react with DNA histone complexes (Fuccillo et al., 1992). If these antibodies are of the IgM class or are IgG antibodies complexed with IgM RF, false-positive results can occur unless each specimen is also tested against a negative-control antigen. Interference by this mechanism can be minimized, but not always eliminated, by extensive purification of the antigen or by using Western blot assays, where the antigen components are separated by gel electrophoresis and blotted prior to IgM testing.

False-negative results can occur in indirect IgM assays if virus-specific IgG antibodies (and to a lesser extent, other Igs) are present in the serum that can compete with IgM for available epitopes on the antigen bound to the solid-phase (Heinz et al., 1981). This can be a particular problem if the specimen is collected late after onset of symptoms, when IgG antibodies have begun to rise, or after repeat infections (as with respiratory syncytial virus [RSV], parainfluenza viruses, and enteroviruses) or reactivations (as with herpesviruses), where an early and enhanced IgG response can obscure the presence of low-level IgM antibodies (Fig. 1).

To address these concerns, serum to be tested by indirect IgM assays should be pretreated by one of the methods previously described to remove interfering IgG antibodies. IgG absorption methods have the advantage of also removing RF by removing RF-IgG complexes, although the efficiency of these methods needs to be determined on a case-by-case basis (Fuccillo et al., 1992). If RF is of particular concern, additional steps can be taken to to reduce its influence (Meurman, 1983), i.e., (i) blocking the binding sites of RF by adding aggregated IgG to the serum diluent, which has a higher affinity for RF than native IgG; (ii) using labeled $F(ab')_2$ fragments as detector antibody, thereby eliminating the Fc portion of the IgG molecule that binds RF; and (iii) determining the optimal dilution of serum that retains assay sensitivity while minimizing background RF binding. Independent of methods used to pretreat serum specimens, all specimens must be run against negative control antigen (e.g., uninfected tissue culture lysate) to reveal nonspecific activity.

The first of the indirect assays to be applied for the detection of virus-specific IgM antibodies was the immunofluorescence assay (IFA) (Baublis and Brown, 1968). In this test, the antigen most often used is infected cells fixed to glass slides. The method is essentially identical to indirect IFA for IgG antibodies, except that fluorescein isothiocyanate-labeled anti-human IgM detector antibody is used. The reading of IFA-IgM tests requires considerable skill and experience. Nonspecific staining may cause false-positive

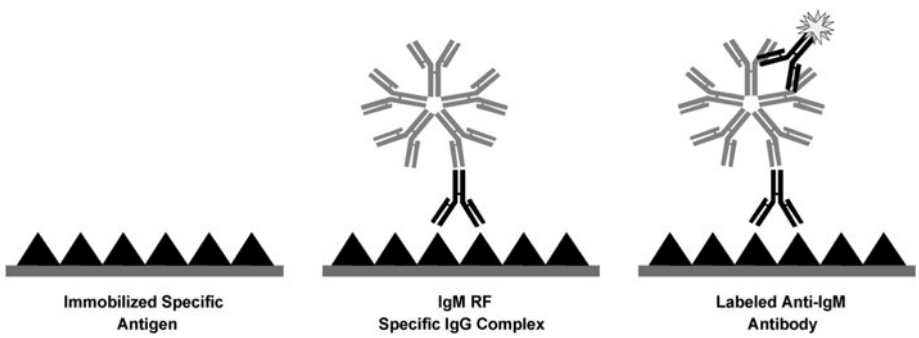

FIGURE 3 Schema of false-positive IgM result caused by RF interference in indirect solid-phase immunoassay.

readings, but an experienced IFA microscopist can minimize false-positive results by differentiating patterns of specific and nonspecific fluorescence, a possibility that does not exist for enzyme immunoassays (EIAs). In experienced hands, and with the use of high-quality reagents, the IFA-IgM test can be both a sensitive and reliable method, but it should not be performed by those laboratories lacking substantial skill and past experience with IFA.

Solid-phase radioimmunoassay, first used to detect viral antibodies in the early 1970s, have been replaced by EIAs for IgM detection in the diagnostic virology laboratory. EIAs were first reported for the detection of virus-specific IgM antibodies by Voller and Bidwell (1976). EIAs offer high sensitivity, require little upfront training to perform, and are easily automated. EIAs are also remarkably flexible as to form and test results and can be read subjectively by eye or objectively by using microplate spectrophotometers linked to computers to aid data analysis.

Reverse, or Capture, IgM Immunoassays

Another approach for avoiding the problems of competitive interference from IgG and nonspecific reactivity seen with the traditional indirect immunoassays previously described is the reverse, or capture, IgM design (Fig. 4). This method, first reported for detecting virus-specific IgM antibodies by Flehmig (1978), and more fully described by Duermeyer et al. (1979), employs a solid-phase surface coated with anti-human IgM antibodies to capture and bind IgM antibodies in the clinical specimen. The washing process removes other Igs and any immune complexes in the specimen. The addition of specific viral antigen, followed by a second, usually enzyme-labeled, antiviral antibody completes the test. This approach has attracted considerable support among virologists, and capture IgM immunoassays have now been described for most of the viruses of public health importance.

Capture immunoassays have proven to be very sensitive and specific for detection of virus-specific IgM antibodies and, where comparisons have been made, are generally superior to indirect assay designs (Heinz et al., 1981; Roggendorf et al., 1981; Gerna et al., 1987; Besselaar et al., 1989; Re and Landini, 1989). Because the first step in the capture assay leads to separation of IgM, competition between IgM and other Igs (primarily IgG) does not occur, and interference with RF is greatly reduced. Nevertheless, there are potential problems with the capture assay design that should be considered. Because virus-specific IgM antibodies must compete with nonspecific IgM for available sites on the capture phase, assay sensitivity can vary with the relative proportion of specific to nonspecific IgM antibodies in the specimen. RF interference still exists with IgM capture assays, although to a lesser extent than with indirect designs. For example, IgM-RF may be captured and bound to the solid phase and then bind to the labeled antiviral antibodies. RF interference by this mechanism can be minimized for capture immunoassays by using the methods described for indirect assays and by (i) substituting monoclonal capture and detector antibodies for animal polyclonal reagents, which appears to reduce unwanted RF activity (Wielaard et al., 1985; Chantler and Evans, 1986), and (ii) eliminating detector antibody entirely through direct labeling of antigen with enzyme (Schmitz et al., 1980; Nielsen et al., 1987; Tuokko, 1988; Morinet et al., 1991) or using erythrocytes if the antigen has hemagglutinating properties (Krech and Wilhelm, 1979; Van der Logt et al., 1981, 1985; Hilfenhaus et al., 1993). A more problematic, but less common, mechanism of RF interference in capture immunoassays is the binding of RF-antiviral IgG complexes to the capture phase, which can mimic specific IgM antibody (Fig. 5). These specimens generally have very high levels of both RF and antiviral IgG antibodies and therefore are less commonly encountered. The main limitations of capture IgM immunoassays for viral diagnosis have been their increased complexity and cost of manufacture, although these assays are now commercially available for many viruses.

Recombinant Protein-Based IgM Assays

An important recent development for IgM and other immunoassays is the availability of defined viral recombinant proteins that resolve some of the problems associated with use of whole virus antigen. Firstly, whole virus antigen produced in tissue culture or suckling mouse brain presents a potential health hazard through exposure to infectious virus requiring use of a biocontainment facility. In addition, production and purification of whole virus antigen is often cumbersome and expensive and may suffer lot-to-lot variation due to many factors including virus strains, cell lines, and cell condition, which may result in differences in the relative proportion of immunoreactive protein and make assay standardization and quantitative interpretation of test results difficult. One approach to address these concerns is to replace the whole virus antigen with recombinant antigen consisting of carefully chosen immunodominant epitopes.

Recombinant protein-based IgM immunoassays have been developed for the diagnosis of many viral infections, including West Nile virus (Muerhoff et al., 2004; Hogrefe et al., 2004), human cytomegalovirus (Landini et al., 1995, Maine et al., 2000), human parvovirus B19 (Pickering et al., 1998), mumps virus (Samuel et al., 2002), dengue virus (Anandarao et al., 2006), severe acute respiratory syndrome (SARS)-associated coronavirus (CoV) (Yu et al., 2007),

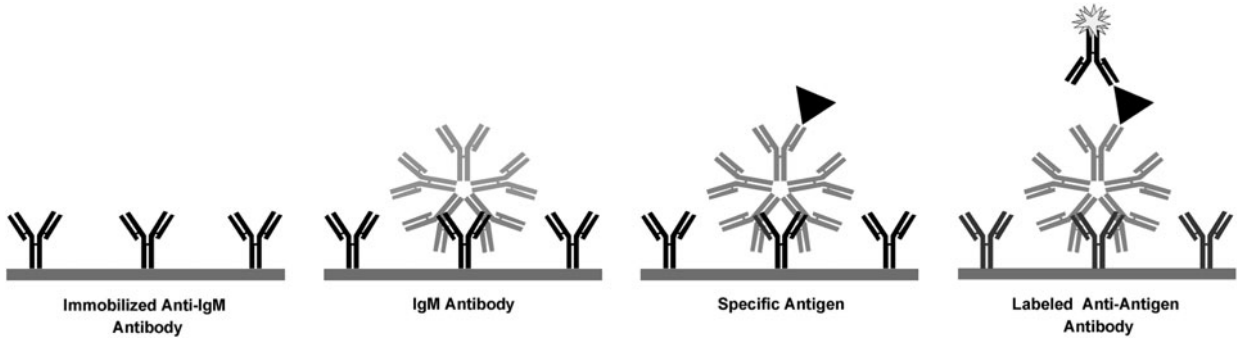

FIGURE 4 Schema of reverse, or capture, solid-phase immunoassay for IgM antibody.

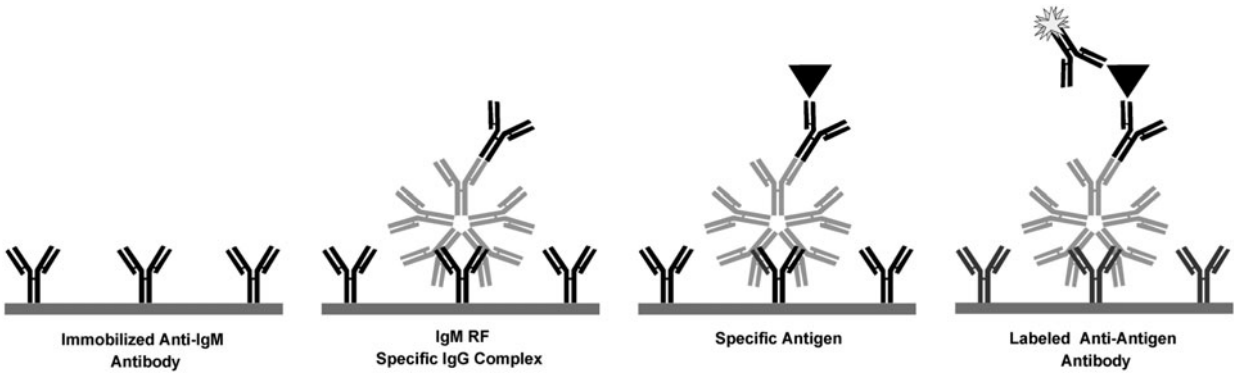

FIGURE 5 Schema of false-positive IgM result caused by RF interference in reverse, or capture, solid-phase immunoassay.

and many others. Compared to the whole virus antigen-based IgM immunoassay, the recombinant protein-based assay offers several distinct advantages. First, the use of infectious virus and special safety precautions used for antigen production are not required. Recombinant proteins are safe to use in laboratories that are not equipped with biocontainment facilities (safety). Second, recombinant proteins can be easily standardized and quality controlled (standardization of antigen production). Third, recombinant antigen production is efficient and relatively economical, thus eliminating the generally high production costs associated with virus cultivation (cost efficiency).

In choosing the appropriate antigen, it is important to choose those that are highly immunogenic and abundantly expressed during virus infection. For example, in hantavirus infection, the nucleocapsid protein induces an early, strong, and long-lasting immune response and is considered an essential antigen for use in recombinant-based hantavirus immunoassays (Schmidt et al., 2005). An appropriate antigen would ideally have a high epitope density, thus increasing assay sensitivity. Also, careful choice of unique virus-specific epitopes will likely improve assay specificity.

Several well-established prokaryotic and eukaryotic expression systems can be used to efficiently express high levels of recombinant proteins. The advantages of using a prokaryotic expression system, such as *Escherichia coli*, to produce recombinant protein may be considerable due to the ease of large-scale production and the low costs involved in growing bacteria. The recombinant protein can be obtained within a relatively short time (within 1 week after cloning), and the expression and purification procedures are simple and easy to perform. The disadvantages of this expression system are as follows: (i) the recovered proteins do not undergo post-translation modification which may affect their antigenicity and confirmation; (ii) many proteins become insoluble, forming inactive aggregates (inclusion bodies) in the host cell instead of native, soluble, and biologically active antigens (Marston, 1986; Rudolph and Lillie, 1996); and (iii) the use of *E. coli*-expressed proteins may require that serum samples be adsorbed to *E. coli* extracts to reduce nonspecific reactivity (Sjolander et al., 1997). Eukaryotic expression systems including yeast, mammalian cells, and insect (baculovirus) cells are ideal for the expression of recombinant proteins that require posttranslation modification, such as glycosylation or protein folding. Further advantages of these systems include high levels of protein expression and ease of purification. The main disadvantage of these systems is that eukaryotic cells grow slower than prokaryotic cells. Also, the glycosylation processing pathways differ in insect cells from those in mammalian cells, which may affect recombinant glycoprotein immunogenicity (Hüser and Hofmann, 2003). Regardless of the expression system used, it is important to evaluate the expressed protein to ensure that it exhibits the antigenic sites and conformation necessary for specific antigen-antibody recognition.

A concern for using recombinant viral antigens in IgM immunoassays is the potential for antigen cross-reactivity to antibodies against other related viruses, resulting in false positivity. One approach to avoid cross-reactivity toward closely related viruses is to use a truncated recombinant protein that lacks the region of potential cross-reactivity. For example, there is a highly conserved region (FYYLGTGP) that occurs in the N-terminal half of all coronavirus nucleocapsid proteins (Rota et al., 2003). The conserved nature of the nucleocapsid likely accounts for reported antigenic cross-reactivity between SARS-CoV and group I coronaviruses (Ksiazek et al., 2003; Sun and Meng, 2004) and the high rates of false positivity with recombinant SARS-CoV nucleocapsid-based immunoassays (Maache et al., 2006; Woo et al., 2004a, 2004b; Yu et al., 2005). To reduce cross-reactivity, Yu et al. (2007) used a recombinant SARS-CoV nucleocapsid protein in which the highly conserved region was deleted as the antigen in a capture IgM immunoassay. With specificity and sensitivity of 100%, as evaluated with sera from healthy donors and SARS patients, the assay using this truncated SARS-CoV nucleocapsid proved to be sensitive and reliable.

Another approach that may minimize cross-reaction entails splicing together several unique virus-specific epitopes (multiepitope) that do not cross-react with antibodies from related viruses. This approach depends on fusing together linear immunodominant epitopes using a flexible linker such as glycine. The resultant epitopes are freely accessible, thus contributing to the overall IgM specificity of the molecule (AnandaRao et al., 2005). Using this approach, AnandaRao et al. (2006) designed a dengue virus multiepitope protein (r-DME-M) consisting of four linear epitopes from nonstructural protein NS1 of dengue serotypes 1 to 4 for use in an indirect IgM immunoassay. This multiepitope recombinant protein was specific for anti-dengue IgM but did not cross-react with other flaviviruses. Also, since this assay used virus-specific IgM epitopes, competing IgG antibodies were not a concern, so r-DME-M can be used as an antigen in an indirect assay format. The multiepitope dengue IgM

immunoassay demonstrated higher sensitivity and specificity than a commercially-available IgM immunoassay (Dengue Due rapid strip test; PanBio, Perth, Australia).

Overall, recombinant protein-based IgM immunoassays have demonstrated comparable and often superior sensitivity and specificity to whole-virus antigen immunoassays (Cuzzubbo et al., 2001; Landini et al., 1995; Sathish et al. 2002; Samuel et al., 2002; Purdy et al. 2004). The approach of creating customized recombinant antigens using either prokaryotic or eukaryotic expression and simple purification offers promising alternative options to serodiagnosis with the potential to eliminate the concerns associated with whole-virus antigen-based assays.

INTERPRETATION OF IgM ASSAY RESULTS

The diagnostic value of specific IgM antibody assays is variable and dependent on the virus and the infection in question. Generally, pronounced transient IgM responses are characteristic of acute virus infections caused by viruses that elicit long-lasting immunity, such as with rubella, measles, mumps, parvovirus B19, and hepatitis A viruses. In these infections, a reliable diagnosis can usually be made by specific IgM antibody testing of a single serum specimen taken early in the illness. In infections with viruses belonging to groups of closely related strains or serotypes (e.g., herpesviruses, adenoviruses, enteroviruses, parainfluenza, and togaviruses), IgM serodiagnosis may be complicated by the possible absence of a specific IgM response, as well as by possible false-positive reactions to related viruses. Because the consequences of misdiagnosis can be severe, it is important to interpret IgM results cautiously and with careful consideration of the clinical presentation of the patient.

False-negative IgM antibody results may occur in a number of clinical settings. The IgM response is typically poor in children less than 3 months of age, as has been shown in studies of RSV infection (Welliver et al., 1980), and tends to be generally weak for many respiratory virus infections (e.g., RSV, parainfluenza viruses, adenoviruses, and influenza viruses), possibly because these infections are localized and less immunogenic or because they represent repeat infections that often yield a diminished IgM response. Immunocompromised persons may fail to generate detectable IgM antibodies or present a delayed response. In rare cases, e.g., aplastic crisis caused by parvovirus B19 infection, onset of symptoms may precede development of IgM antibodies and therefore require a different diagnostic method, e.g., PCR assay. There can also be considerable individual variation in the appearance of IgM antibodies after the onset of symptoms among heterogeneous populations, as has been demonstrated for rubella infection (Meurman, 1983).

False-positive IgM antibody results may occur due to cross-reactions between closely related viruses. Such cross-reactions have been reported for togavirus (Wolff et al., 1981), coxsackie B virus (McCartney et al., 1986), some herpesviruses (Miendje Deyi et al., 2000), and parainfluenza virus infections (Vuorimen and Meurman, 1989). Moreover, evidence suggesting the occurrence of true polyclonal IgM production in cases of acute infectious mononucleosis also has been reported (Morgan-Capner et al., 1983). Their data suggest that production of various IgM antibodies may result from Epstein Barr virus-induced stimulation of B lymphocytes already committed by prior antigenic stimulation. Simultaneous IgM reactivity by this or other mechanisms also have been reported to occur between IgM tests for rubella, measles, and parvovirus B19 (Kurtz and Anderson, 1985; Jenkerson et al., 1995; Thomas et al., 1999). In general, however, the heterologous IgM antibody responses are low compared with homologous titers. Nevertheless, these observations emphasize the importance of careful interpretation of positive virus-specific IgM tests together with the complete clinical picture.

Finally, the expected duration of the IgM response must be considered when interpreting the significance of a positive test result. For the diagnosis of an acute infection, the ideal maximum duration of specific IgM antibodies should be 1 to 3 months. Variations in the temporal appearance of IgM antibodies, including the occurrence of prolonged IgM antibody responses, can result in difficulties in interpreting the significance of the test results in relation to the clinical illness in question. Generally, the IgM antibody response following an acute virus infection is of limited duration. However, persistent IgM antibodies have been observed in complicated infections (e.g., post-measles encephalitis), chronic infections (e.g., hepatitis B and parvovirus B19), congenital infections (e.g., rubella and parvovirus B19), and some immunosuppressed patients (e.g., cytomegalovirus). The persistence of specific IgM in these cases appears to be related to the persistence of viral antigen (or even replicating virus) in the patient. Occasionally, prolonged IgM antibody responses have been observed without any apparent reason (e.g., West Nile virus). Also, as more-sensitive methods for IgM determination are developed, the time following an acute infection during which specific IgM is detectable will increase. In such cases, collection of a second serum specimen later in the course of the illness to demonstrate increasing antibody titer or use of IgG antibody avidity testing can help resolve indeterminate IgM test results (Gutierrez and Maroto, 1996).

CONCLUSION

Sensitive and reliable methods for the determination of virus-specific IgM antibodies have been developed for most human viral pathogens, and commercial reagents and

TABLE 2 A selection of commercial vendors of diagnostic kits for virus-specific IgM antibodies

Virus(es)	Vendor(s)[a]
Cytomegalovirus	3, 5, 6, 7, 10, 12, 13, 14
Dengue virus, West Nile virus, hantavirus, and other arboviruses	5, 6, 8, 9, 11
Epstein-Barr virus	5, 6, 7, 10, 12, 14
Hepatitis A and B viruses	1, 2, 7
Human herpesviruses 1 and 2	3, 4, 5, 6, 7, 10, 12, 13, 14
Human herpesvirus 6	11
Measles virus	6, 7, 9, 12, 14
Mumps virus	5, 7, 12
Parvovirus B19	4, 6, 8
Rubella virus	3, 4, 5, 6, 7, 12, 13, 14
Varicella-zoster virus	5, 6, 7, 10, 12, 14

[a]1, Abbott Laboratories, Abbott Park, IL; 2, BioChain, Hayward, CA; 3, Bio-Check, Foster City, CA; 4, Biotrin International, Dublin, Ireland; 5, Calbiotech, Spring Valley, CA; 6, Dade Behring, Deerfield, IL; 7, DiaSorin, Stillwater, MN; 8, Focus Diagnostics, Cypress, CA; 9, Millipore (Chemicon International), Temecula, CA; 10, Meridian Bioscience, Cincinnati, OH; 11, Panbio, Columbia, MD; 12, Trinity Biotech, James Town, NY; 13, United Biotech, Mountain View, CA; 14, Wampole Laboratories, Princeton, NJ.

complete diagnostic kits are available for many virus infections (Table 2). Substantial improvements in assay design and reagent quality have occurred during the past three decades, and continued improvements can be expected with the development and wider availability of well-defined recombinant and synthetic viral antigens and novel solid-phase platforms. These methods, when adopted for routine use in clinical laboratories, should bring considerable improvement to viral diagnostic services.

Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention or the U.S. Department of Health and Human Services.

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Susceptibility Test Methods: Viruses†

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12

During the past decade and a half, safe and effective antiviral therapy has been developed for treatment of a number of viral infections (Beutner, 1995; Cirelli, et al., 1996; Coen and Richman, 2007). Great strides have been made, most notably in the development of antiviral agents for treatment of human immunodeficiency virus (HIV). While the overwhelming majority of clinical virus isolates from drug-naive patients are susceptible to antiviral agents, widespread use of some antiviral agents has led to the emergence of drug-resistant strains, particularly in immunocompromised hosts (Bean et al., 1987; Belshe et al., 1989; Belshe et al., 1988; Berkowitz and Levin, 1985; Biron, 1991; Englund et al., 1990; Lok, 2007). Diagnostic virology laboratories are increasingly asked to perform in vitro testing of antiviral agents when patients fail to respond clinically to antiviral therapy. This chapter discusses the clinical situations in which antiviral resistance has emerged, thus necessitating in vitro susceptibility testing and an overview of the phenotypic and genotypic susceptibility testing methods that have been employed to detect resistance.

CLINICAL INDICATIONS FOR ANTIVIRAL SUSCEPTIBILITY TESTING

Antiviral susceptibility testing is essential for defining mechanisms of antiviral resistance, for determining the frequency with which drug-resistant viral mutants emerge in clinical practice, to test for cross-resistance to alternative agents, and when evaluating new antiviral agents. With the exception of antiviral susceptibility testing for herpes simplex virus (HSV), results of in vitro phenotypic assays are usually not available in a timeframe relevant for patient management. Nevertheless, clinical deterioration of patients undergoing antiviral therapy can be associated with resistant virus. Results of antiviral susceptibility testing may be helpful in certain clinical situations. Persistent or worsening HSV or varicella-zoster virus (VZV) infection while on acyclovir may indicate drug resistance. Alternative therapy such as foscarnet and cidofovir are available. Furthermore, HSV or VZV causing recurrent infection is often thymidine kinase (TK) competent and therefore susceptible to acyclovir, which has minimal toxicity compared to foscarnet. Persistent or worsening human cytomegalovirus (HCMV) retinitis, pneumonitis, or colitis unresponsive to ganciclovir may also indicate drug-resistant virus. Cidofovir and foscarnet can be used as alternative agents. Influenza A virus isolates resistant to amantadine and rimantadine readily emerged during clinical trials of these drugs; therefore, continuous shedding or transmission of influenza A virus in a population which is being prophylaxed or treated with these agents may be due to drug resistance. Prolonged influenza virus shedding during therapy with a neuraminidase (NA) inhibitor (NI) may be due to emergence of NI-resistant virus. Monitoring for HIV type 1 (HIV-1) antiretroviral resistance is essential for assessing failure of a particular regimen to suppress HIV replication and to test for cross-resistance to alternative antiretroviral drugs to aid in selection of appropriate salvage therapy. Guidelines developed by the U.S. Department of Health and Human Services (available at http://aidsinfo.nih.gov [accessed 6 August 2007]) and by a European panel (Vandamme et al., 2004) have recommended that resistance testing be performed for persons with acute and chronic HIV infection prior to initiation of therapy, when changing antiretroviral regimens in cases of virologic failure, and in cases of suboptimal viral load reduction after institution or modification of therapy. Baseline resistance testing is recommended because transmission of drug-resistant HIV strains has been documented and has been associated with suboptimal response to therapy (Little et al., 2002). A genotypic assay is recommended when testing antiretroviral-naive persons because of its more rapid turnaround time. In cases of suboptimal viral load reduction, there may be resistance to only one component of the combination therapy; substitution of an alternative drug may provide clinical benefits, although, in general, at least two fully active agents should be added to a failing regimen. Prolonged treatment of chronic hepatitis B virus (HBV) infections selects for antiviral resistance at varying rates, depending upon the particular agent used. Resistance testing of treated patients with HBV virologic breakthrough after treatment with nucleoside/nucleotide analogs is recommended (Lok, 2007; Lok and McMahon, 2007).

DEFINITION OF ANTIVIRAL RESISTANCE

Antiviral resistance is a decrease in susceptibility to an antiviral drug that can be clearly established by in vitro testing and can be confirmed by genetic analysis of the virus and biochemical study of the altered enzymes. In vitro drug

†This chapter contains information presented in the *Manual of Clinical Microbiology*, 9th ed. (Arens and Swierkosz, 2007).

resistance must be distinguished from clinical resistance, in which the viral infection fails to respond to therapy. Clinical failures may or may not be due to the presence of a drug-resistant virus. Failure to achieve clinical response also hinges on other factors such as the patient's immunologic status and the pharmacokinetics of the drug in that individual patient. For example, limited penetration of drug into the central nervous system may allow escape of HIV-1 despite suppression of the virus at other sites. Poor oral absorption and binding to plasma proteins may limit the bioavailability of certain drugs. Furthermore, administration of certain antiretroviral drugs in combination may interfere with absorption or stimulate elimination of one or more of the coadministered drugs. Patient-specific factors such as nonadherence, intolerance to an antiretroviral drug, or an intercurrent infection can also lead to increases in HIV-1 plasma viremia despite in vitro susceptibility (http://aidsinfo.nih.gov).

VARIABLES OF ANTIVIRAL SUSCEPTIBILITY TESTING

To date, few standards exist for antiviral susceptibility testing. The Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards or NCCLS) recently published an approved standard for susceptibility testing of HSV as a first step in developing consensus protocols for antiviral susceptibility testing (NCCLS, 2004). The major obstacle to standardization of antiviral susceptibility testing is that many variables influence the final result. These include (i) cell line, (ii) viral inoculum titer, (iii) incubation time, (iv) concentration range of the antiviral agent tested, (v) reference strains, (vi) assay method, (vii) endpoint criteria, (viii) calculation of endpoint, and (ix) interpretation of endpoint. Testing of a single virus isolate may lead to greatly different endpoints depending on the type of cell culture used (De Clerq, 1982; Harmenberg et al., 1980; Leary et al., 2002). For example, the activity of acyclovir appeared greater than that of penciclovir when a plaque-reduction assay (PRA) was performed in Vero cells; the converse was true when the assay was performed in WI-38, VA-13, and WISH cell lines. Both drugs had comparable activity when tested in A549 cells (Leary et al., 2002). The titer of the virus inoculum is also critical; too large an inoculum can make a susceptible isolate appear resistant; too small an inoculum can make all isolates appear susceptible (Harmenberg et al., 1980). The length of time that virus incubates in the presence of drug must be sufficient to allow detection of small plaques, in the case of a PRA, or to allow growth of a subpopulation of resistant virus which may replicate at a slower rate than that of wild-type virus (Baldanti et al., 1996). The prolonged incubation times of peripheral blood mononuclear cell (PBMC)-based assays for HIV-1 susceptibility testing have been shown to select for subpopulations of HIV-1 variants not present in the starting inoculum (Kusumi et al., 1992). Moreover, different assay methods can produce different results. For example, the dye uptake (DU) assay for HSV susceptibility testing produces 50% inhibitory concentrations (IC_{50}s) higher than those produced by PRA (Hill et al., 1991a). The concentration range of the drug tested affects the quality of the dose-response curve and, therefore, the validity of endpoint calculations. Susceptibility results are usually expressed as IC_{50}s because of the greater mathematical precision of the 50% endpoint than of a 90 or 99% endpoint. (Synonyms for IC_{50} are 50% inhibitory concentration and 50% effective concentration.) However, debate continues concerning the appropriate endpoint, i.e., 50% versus 90 or 99% inhibition. IC_{50}s are more precise and reproducible but IC_{90}s may correlate better with clinical response and are better at detecting subpopulations of drug-resistant strains among sensitive ones (NCCLS, 2004; Dekker et al., 1983). Moreover, few studies have correlated in vitro results with clinical response (Bean et al., 1987; Erice et al., 1989; Jacobson et al., 1990; Lehrman et al., 1986; Richman, 1995; St. Clair et al., 1993).

Another critical variable is the heterogeneity within the population of a virus "isolate." A single clinical isolate actually represents a mixture of drug-susceptible and drug-resistant phenotypes (Baldanti et al., 1998; Hill et al., 1991b; Richman et al., 1991; St. Clair et al., 1991). A virus population that has never encountered an antiviral agent is predominantly drug susceptible; resistant virus may be present at low levels. The presence of low levels of resistant virus in a population that is predominantly drug susceptible might not be reflected in the IC_{50} but would manifest its presence in higher IC_{90}s or IC_{99}s. At this time, it is unknown whether a small fraction of drug-resistant virus is important to the behavior of the virus in vivo or how such a fraction might affect the response of the infection to therapy in an otherwise healthy host. However, in immunocompromised patients, under the continued selective pressure of antiviral therapy, resistant virus can emerge, and its presence can often be correlated with progressive viral disease (Bean et al., 1987; Chatis and Crumpacker, 1992; Drew et al., 1991; Englund et al., 1990; Erice et al., 1989; Jacobson et al., 1990; Pahwa et al., 1988; Safrin et al., 1990; Safrin et al., 1991a; Safrin et al., 1991b; Sibrack et al., 1982).

The genetic locus at which a mutation occurs also affects susceptibility testing endpoints. For example, DNA polymerase mutations of HSV, VZV, and HCMV usually confer relatively smaller increases in in vitro resistance than do TK mutations, which could go undetected in a mixed population of wild-type and mutant virus. In HIV-1, the level of in vitro resistance to protease inhibitors (PIs) increased as the number of protease gene (PR) mutations increased (Hertogs et al., 1998). A good definition of such mixtures can be obtained only by testing appropriate concentrations of drug and a sufficiently large fraction of the population to detect resistant strains.

TESTING METHODS

Phenotypic Versus Genotypic Assays

Phenotypic assays are in vitro susceptibility assays that measure the inhibitory effect of antiviral agents on the entire virus population in a patient isolate. A variety of endpoint measurements have been utilized, including a reduction in the number of plaques, inhibition of viral DNA synthesis, reduction in the yield of a viral structural protein, e.g., hemagglutinin of influenza or p24 antigen of HIV, or reduction in the enzymatic activity of a functional protein, e.g., HIV-1 reverse transcriptase (RT) or influenza virus neuraminidase. Phenotypic assays in use include PRA, DU, DNA hybridization, enzyme immunoassay (EIA), neuraminidase inhibition, and yield-reduction assays for herpes group and influenza A viruses and PBMC cocultivation and recombinant virus assays for HIV-1. Genotypic assays analyze viral nucleic acid to detect specific mutations that cause antiviral drug resistance. Genotyping has been applied primarily to HBV, HCMV, and HIV-1. Genotypic assays include DNA sequencing by automated sequencers, PCR amplification

and restriction enzyme digestion of the products, and hybridization to microarrays of oligonucleotide probes. Phenotypic and genotypic assays have unique features that complement each other. Phenotypic assays are better suited to assess the combined effect of multiple resistance mutations on drug susceptibility. This is especially important for viruses such as HBV, HCMV, and HIV-1, which acquire resistance-associated mutations in multiple genes that may manifest as new patterns of resistance, cross-resistance, multidrug resistance, or even reversal of resistance (Hertogs et al., 1998; Iversen et al., 1996; Shaw et al., 2006; Smith et al., 1997; St. Clair et al., 1991). However, most phenotypic assays are labor-intensive, expensive, and have a long turnaround time. Genotypic assays are relatively inexpensive and have shorter turnaround times but cannot detect mutations outside the selected target. Interpretation of genotypic assays is problematic due to the complex interactions of resistance mutations, which result in a particular drug resistance phenotype. The major phenotypic and genotypic assays in use are discussed below.

Control Strains

Simultaneous testing of control strains is crucial when antiviral susceptibility testing is being done. Reference strains should include genetically and phenotypically well-characterized drug-susceptible and drug-resistant isolates. Drug-resistant strains chosen for reference should include those with drug resistance phenotypes relevant to the mode of action of the drug to be tested. For example, for testing nucleoside analogs, which require phosphorylation by viral TK, e.g., acyclovir versus HSV and VZV, TK-negative or -deficient strains should be included. Susceptibility testing of HCMV should include both UL97 and UL54 mutants. For HIV-1 testing, mutants resistant to both nucleoside analog and nonnucleoside analog inhibitors of RT, and mutants resistant to PIs should be used. The National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program (Bethesda, MD) provides upon request a number of reference strains of HSV, VZV, and HCMV, including the drug-resistant strains mentioned above and laboratory control strains HCMV AD169 and VZV Oka. For susceptibility testing of HIV-1, the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program has a repository of strains with various resistance phenotypes. Pharmaceutical companies and the American Type Culture Collection are also sources of control strains.

PRA for CMV, HSV, and VZV

The PRA has classically been the "standard" method of antiviral susceptibility testing to which new methods are compared (Biron and Elion, 1980; Biron et al., 1985; Hayden et al., 1980; Hill et al., 1991a; McLaren et al., 1983). Because many variations of the PRA have been reported, the CLSI developed a standard for PRA testing of HSV (NCCLS, 2004). Likewise, the HCMV Resistance Working Group of the AIDS Clinical Trials Groups has formulated a standardized PRA for HCMV susceptibility testing (Landry et al., 2000). The challenges associated with HCMV susceptibility testing by PRA became apparent when, despite adherence to a consensus PRA protocol, some collaborating laboratories had difficulty distinguishing drug-susceptible and drug-resistant HCMV isolates.

The principle of the PRA is the inhibition of viral plaque formation in the presence of antiviral agent. The concentration of antiviral agent inhibiting plaque formation by 50% is considered the IC_{50}. Although the PRA is tedious and consumes more reagents than other methods, it is appropriate for small-scale testing of isolates. Prior to performing the antiviral susceptibility assay per se, titers of HSV isolates must be determined to ensure an inoculum appropriate for the surface area of the assay wells or plates (i.e., approximately 100 PFU/60-mm-wide tissue culture plate). Because clinical strains of HCMV and VZV are cell associated and because low-titer cell-free stocks are less stable during storage, infected cell suspensions (obtained by trypsin treatment of the infected monolayer) can be conveniently used for these viruses. Two to three passages of clinical strains of HCMV and VZV in cell culture are usually necessary to obtain a sufficient titer of virus. Low-passage isolates should be used because they are more likely to be representative of the original mixed population of the clinical isolate than a higher-passage stock would be. Well-characterized drug-susceptible and drug-resistant strains of HSV, HCMV AD169, and VZV Oka or Ellen serve as reference strains. Stepwise instructions for performance of the PRA for HSV, VZV, and HCMV have been published elsewhere (NCCLS, 2004; Hill et al., 1991a; Swierkosz and Biron, 1994; Swierkosz and Biron, 1995).

A modified PRA has been described that utilizes Vero or CV-1 cells that have been stably transformed with the *Escherichia coli lacZ* gene under the control of an HSV type 1 early promoter which express beta-galactosidase only after infection with HSV. Plaques were visualized after histochemical staining for beta-galactosidase (Tebas et al., 1998; Tebas et al., 1995). Proposed susceptibility breakpoints determined by the PRA are listed in Table 1.

DU Assay

The DU assay has been used for many years for susceptibility testing of HSV (Hill et al., 1991a; McLaren et al., 1983). This assay is based on the preferential uptake of a vital dye (neutral red) by viable cells but not by nonviable cells. The extent of viral lytic activity is determined by the relative amount of dye bound to viable cells after infection with HSV compared with the amount bound to uninfected cells. The dye bound by viable cells is eluted by ethanol and measured colorimetrically. The drug concentration inhibiting viral lytic activity by 50% is considered the IC_{50}.

The DU assay consistently gives IC_{50}s of acyclovir that are three to five times greater than those given by PRA. This difference is most likely due to the higher inoculum used in the DU assay (500 PFU/ml) and to the use of a liquid overlay, which allows drug-resistant virus to "amplify," thus resulting in a more sensitive detection of small amounts of drug-resistant virus. Therefore, the DU assay uses a cutoff IC_{50} of >3 μg/ml to denote acyclovir resistance (Table 1).

Advantages of the DU method include its ability to be semiautomated, allowing for efficient testing of large numbers of isolates and its ability to detect smaller amounts of resistant virus than the PRA can detect. Disadvantages are the relatively high cost of automated equipment and the technical problems caused by overseeding of cells into the culture wells and precipitation of neutral red onto the monolayer. The stepwise procedure for the DU assay has been previously published (Hill et al., 1991a; McLaren et al., 1983).

DNA Hybridization

DNA hybridization assays have been used to measure the effect of different antiviral compounds on DNA synthesis. These methods semiquantitatively measure the amount of

TABLE 1 Proposed guidelines for antiviral susceptibility results of herpes group and influenza viruses

Virus(es)	Antiviral agent	Method	IC_{50} denoting resistance	Reference(s)
HSV	Acyclovir	PRA	≥2 μg/ml	McLaren et al., 1983
		DNA hybridization	≥2 μg/ml	Englund et al., 1990; Swierkosz et al., 1987
		DU	≥3 μg/ml	Hill et al., 1991a; McLaren et al., 1983
	Famciclovir (active metabolite = penciclovir)	PRA and DNA hybridization	Definitive breakpoints cannot be established	Leary et al., 2002; Standring-Cox et al., 1996
	Foscarnet	PRA	>100 μg/ml	Safrin et al., 1990
	Vidarabine	PRA	≥2-fold increase of IC_{50} compared to control or pretherapy isolate	Safrin et al., 1991b
VZV	Acyclovir	PRA and DNA hybridization	≥3- to 4-fold increase of IC_{50} compared to pretherapy isolate or to control strain	Biron and Elion, 1980; Jacobson et al., 1990; Safrin et al., 1991a
	Famciclovir	PRA and DNA hybridization	Definitive breakpoints cannot be established	Standring-Cox et al., 1996
	Foscarnet	Late antigen reduction assay	300 μM	Safrin et al., 1991a
HCMV	Cidofovir	PRA and DNA hybridization	>2 μM	Chou et al., 1995b; Erice et al., 1997; Smith et al., 1997
	Foscarnet	PRA and DNA hybridization	>400 μM	Chou et al., 1995b; Erice et al., 1997
			>324 μM	Smith et al., 1997
	Ganciclovir	PRA and DNA hybridization	≥3- to 4-fold increase of IC_{50} compared to pretherapy isolate or control strain (≈3 μg/ml)	Drew et al., 1991; Erice, 2000; Pepin et al., 1992
			>8 μM	Smith et al., 1997
Influenza A virus	Amantadine, rimantadine	EIA	>0.1 μg/ml	Belshe et al., 1988; Belshe et al., 1989
Influenza A and B viruses	Oseltamivir and zanamivir	NI assay	>1,000-fold decrease in NA activity	Gubareva et al., 1998; Gubareva et al., 2001

viral DNA produced in the absence and presence of antiviral drug, and IC_{50}s are calculated from these data. Good correlation between the PRA and a dot blot hybridization assay has been demonstrated (Biron et al., 1986; Gadler, 1983). The stepwise procedure has been detailed previously (Swierkosz and Biron, 1995). DNA-DNA hybridization test kits previously commercially available (Hybriwix Probe Systems, Diagnostic Hybrids, Inc., Athens, OH) have been used successfully for susceptibility testing of HSV, HCMV, and VZV (Chou et al., 1995a; Chou et al., 1995b; Chou et al., 1997; Dankner et al., 1990; Englund et al., 1990; Jacobson et al., 1990; Safrin et al., 1990; Safrin et al., 1991a; Safrin et al., 1991b; Smith et al., 1997; Swierkosz et al., 1987). The Hybriwix assays are no longer commercially available.

EIA

EIAs have been developed for susceptibility testing of HSV, VZV, and influenza A virus (Belshe et al., 1989; Berkowitz and Levin, 1985; Rabalaiss et al., 1987; Safrin et al., 1996). EIA permits quantitative measurement of viral activity by spectrophotometric analysis; IC_{50}s are calculated as the concentrations of antiviral agent that reduce the absorbance to 50% of that of the virus control. EIA is more suited than the PRA and DU assay to the routine diagnostic laboratory. Susceptibility results for HSV and VZV determined by this method have correlated well with those obtained by PRA.

Susceptibility testing of influenza A virus by PRA is tedious and labor-intensive. EIA is technically easier and is more suitable for the testing of multiple isolates (Belshe et al., 1989). The EIA utilizes antibodies to influenza A virus hemagglutinins (H1 or H3); viral hemagglutinin expression correlates with viral growth. Amantadine and rimantadine activities are measured by inhibition of hemagglutinin expression. Amantadine- or rimantadine-susceptible and -resistant isolates, whose M2 gene sequences are known, serve as controls and must be tested in parallel with patient isolates. A protocol for the EIA for susceptibility testing of influenza A virus has been published (Swierkosz and Brion, 1994). There are no commercial EIAs available.

Flow Cytometry

Flow cytometry has been applied to susceptibility testing of HSV and HCMV (Lipson et al., 1997; McSharry et al., 1998; Pavic et al., 1997). While the IC_{50}s measured by flow

cytometry were numerically different than those determined by plaque reduction, drug-susceptible isolates could be readily distinguished from drug-resistant isolates. Advantages of antiviral susceptibility testing by flow cytometry include the potential for automation, the objectivity of the assay, and a shorter turnaround time relative to the PRA.

Neuraminidase Inhibition Assay

Recently, two inhibitors of influenza virus NA, oseltamivir and zanamivir, have been approved for treatment of influenza A and B virus infections (Centers for Disease Control and Prevention, 1999). Because there are limited data concerning the emergence of resistant virus in vivo, surveillance is important to determine the frequency with which resistance to NI emerges during active influenza virus infection. Susceptibility studies to date have indicated that assay of NA activity was the best predictive in vivo response to NI (Gubareva et al., 2001; Mendel and Sidwell, 1998). NA activity is assayed using solubilized supernatant from viral culture as the source of viral NA. After incubation of the viral NA with varying concentrations of NI, a fluorogenic substrate is added. Fluorescence is quantitated by a fluorimeter, and the IC_{50} is calculated relative to the activity of viral NA in the control reaction (no NI). A zanamivir-resistant influenza B virus isolate from an immunocompromised child and oseltamivir-resistant influenza A virus (H1N1) isolates from experimentally infected volunteers have been recovered (Gubareva et al., 2001; Gubareva et al., 1998).

Yield Reduction Assay

The yield reduction assay reflects the ability of an antiviral agent to inhibit the production of infectious virus, rather than the formation of a plaque. Cell monolayers are infected with virus, incubated in the presence of antiviral compound, and then lysed. Cell-free virus titers are subsequently determined by plaque assay. The endpoint is defined as the concentration of antiviral agent that reduces virus yield by 50% in comparison with that of untreated control cultures. When used for susceptibility testing of HSV against penciclovir and acyclovir, the IC_{50}s for penciclovir were equivalent to or lower than those for acyclovir (Boyd et al., 1993; Leary et al., 2002). The greater activity of penciclovir is postulated to be the result of the extended half-life of penciclovir triphosphate (Boyd et al., 1993).

Measurement of HSV and VZV TK Activity by Plaque Autoradiography

Functional viral TK is required for initial phosphorylation of acyclovir. To determine whether resistance to acyclovir is due to diminished or altered viral TK, two plaque autoradiograph methods are used (Martin et al., 1985). Incorporation of [^{125}I]iododeoxycytidine (IdC), a pyrimidine analog selectively phosphorylated by the VZV- or HSV-specific TK, correlates well with the acyclovir-phosphorylating potential of HSV and VZV isolates. Incorporation of [^{14}C]thymidine (dT) specifically assesses the thymidine-phosphorylating activity of these isolates and is useful for analyzing resistance to pyrimidine nucleoside analogs (acyclovir is a purine nucleoside analog). Most acyclovir-resistant HSV and VZV isolates fail to incorporate both substrates due to diminished TK activity (TK^-); occasionally, strains with altered substrate (TK^a) activity are seen that fail to incorporate IdC but are able to incorporate dT. For IdC incorporation, Vero cells (HSV) and MRC-5 cells (VZV) are used. For dT incorporation, $LMTK^-$ TK^- mouse LM cells (Roswell Park Memorial Institute, Buffalo, NY) are used for HSV; the TK^- cell line 143B is used for VZV dT incorporation. These assays provide both quantitative and qualitative evaluations of the TK status of a mixed population of TK^+ and TK^-. The IdC and dT plaque autoradiograph methods have been detailed elsewhere (Martin et al., 1985; Swierkosz and Biron, 1995).

Assays for HIV

A number of phenotypic assays are in use for susceptibility testing of HIV-1 isolates to nucleoside analog RT inhibitors (Hertogs et al., 1998; Japour et al., 1993; Kellam and Larder, 1994; Larder et al., 1989; Martinez-Picado et al., 1999b; Petropoulos et al., 2000; Shi and Mellors, 1997; St. Clair et al., 1991; Walter et al., 1999; Wilson et al., 2000). A serious limitation of some of these procedures is that not all clinical isolates grow in the cell culture lines used in these assays. The AIDS Clinical Trials Group developed an assay performed in PBMCs that allowed growth of almost all clinical isolates of HIV-1 (Japour et al., 1993). Viral activity is quantitated by measurement of p24 antigen of HIV-1. The PBMC assay, however, is labor-intensive, costly, and difficult to control because of the many variables of the assay, and it has a long turnaround time (weeks). Moreover, this assay requires cocultivation of infected PBMCs with uninfected donor PBMCs to produce a stock of the clinical isolate being tested, which has been shown to select for subpopulations of HIV-1 not present in the original isolate (Kusumi et al., 1992).

A new generation of phenotypic assays, recombinant virus assays (RVAs), has been developed to circumvent these problems (Hertogs et al., 1998; Kellam and Larder, 1994; Martinez-Picado et al., 1999b; Petropoulos et al., 2000; Shi and Mellors et al., 1997). RVA involves RT-PCR amplification of complete RT and PR gene coding sequences directly from the patient's plasma. The amplified RT and PR gene sequences from the patient strain are ligated into a viral vector containing a luciferase gene and then cotransfected along with a plasmid that expresses murine leukemia virus envelope proteins into a suitable receptive cell line. These cells then contain the patient's RT and PR gene-coding sequences in a background of an HIV-1 strain from which the original RT and PR sequences had been deleted. The susceptibilities of the chimeric pseudotyped viruses to all clinically available RT and PR inhibitors are subsequently determined in a single assay in which the ability of virus particles to replicate in the presence of various levels of antiretroviral drug is measured by detection of luciferase activity in the target cells. RVAs thus allow determination of the phenotypic resistance patterns of circulating virus in vivo and circumvent the problem of selection of nonrepresentative variants during cultivation. The RVA can be completed in approximately 10 days from the time of cotransfection (Hertogs et al., 1998; Kellam and Larder, 1994; Martinez-Picado et al., 1999b; Petropoulos et al., 2000; Shi and Mellors et al., 1997). Two RVAs are commercially available, the Antivirogram assay (developed by Virco, Mechelen, Belgium, and available in the United States at Laboratory Corporation of America) (Hertogs et al., 1998) and the PhenoSense assay (Monogram Biosciences, South San Francisco, CA) (Petropoulos et al., 2000). A recent comparison by Zhang et al. (2005) of these two phenotype assays concluded that the PhenoSense results are more precise (i.e., show lower variability) and the PhenoSense assay was more likely to detect resistance to abacavir, didanosine, and stavudine. Limitations of RVAs are the necessity for a minimum of 500 to 1,000 copies of HIV-1 RNA/ml plasma, a lack of consensus as to the appropriate increase in IC_{50} for

each drug that correlates with clinical resistance, and uncertainty about the proportion of the total population of virus that a subpopulation of resistant virus must achieve to be detectable by these assays (Hanna and D'Aquila, 2001; Hertogs et al., 1998).

Genotypic Assays

The genetic basis for antiviral resistance has been extensively studied for HBV, HCMV, and HIV-1. Although not all resistance-associated mutations are known, the majority have been elucidated, allowing the application of molecular diagnostic methods. The major advantage of genotypic assays is the relatively rapid turnaround time compared to phenotypic assays.

HCMV

Genotypic assays have been used to screen HCMV isolates for mutations associated with ganciclovir resistance. Both UL97 (phosphotransferase) and UL54 (DNA polymerase) mutations can be detected; UL97 mutations are responsible for most ganciclovir resistance found to date (Smith et al., 1997). A number of approaches have been successfully applied to genotyping HCMV.

PCR amplification of short fragments of the UL97 gene followed by restriction endonuclease digestion has been used to detect mutations at positions 460, 520, 594, and 595 (Chou et al., 1995a; Chou et al., 1995b; Erice, 1999, 2000). In one study, this assay detected 78% of ganciclovir-resistant UL97 mutants (Chou et al., 1995b). PCR amplification followed by restriction digestion could recognize mutant virus when present at 10% of the total virus population (Smith et al., 1997). The major advantage of this PCR-restriction endonuclease method is the speed with which UL97 mutations can be identified, since HCMV sequences can be directly amplified from many clinical samples. However, the absence of mutations at these key codons in UL97 does not necessarily exclude ganciclovir resistance, as it has been shown that resistance can be attributed to other mutations in UL97 or to mutations in UL54 alone (Erice et al., 1997).

PCR amplification and sequencing of nearly the entire UL54 gene and the fragment of the UL97 gene spanning the conserved domains of the phosphotransferase should theoretically detect all the mutations currently known to confer resistance to ganciclovir (Baldanti et al., 1996; Chou et al., 1995b; Erice et al., 1997; Smith et al., 1997). Wolf et al. (1995) identified a complete set of overlapping primers to be used for sequencing the full UL97 gene. UL97-associated ganciclovir resistance mutations also have been detected directly in patient blood and cerebrospinal fluid (Boivin et al., 1996; Spector et al., 1995; Wolf et al., 1995).

Sequencing of the UL54 gene, for genotypic detection of resistance to ganciclovir, foscarnet, and cidofovir is a large task not generally undertaken by the routine clinical virology laboratory. The UL54 codons that confer resistance to all three anti-HCMV drugs are between codons 408 and 841, inclusive (Erice, 1999; Lurain et al., 1992). Thus, one must amplify a fragment of about 1,500 bp and use 3 or 4 sequencing primers in each direction to obtain a reliable sequence. In a study by Smith et al. (1997), UL97 mutations could be detected in 89% of ganciclovir-resistant isolates, whereas UL54 mutations were present in all high-level ganciclovir-resistant isolates. A caveat that applies to genotypic analysis is that not every mutation is a cause of antiviral resistance. Marker transfer experiments must be performed to definitively determine that a particular mutation is associated with drug resistance. For this purpose, PCR-amplified UL97 and UL54 fragments containing resistance-associated mutations were cotransfected with HCMV strain AD169 (drug susceptible). The resulting recombinant plaques were assayed for antiviral susceptibility by PRA. To further verify transfer of the mutations in question, sequencing was performed across the transfected fragment (Baldanti et al., 1996; Chou et al., 1997).

The field of HCMV genotyping for detection of drug resistance is still evolving. A standardized protocol for sequencing of the UL97 gene was developed by members of the AIDS Clinical Trials Group HCMV Laboratories and published several years ago (Lurain et al., 2001). Nevertheless, the discovery of new mutations that confer drug resistance is ongoing, as evidenced by the recent description of a deletion at UL97 codon 601 that was responsible for the early development of ganciclovir resistance in a renal transplant recipient (Hantz et al., 2005). A recent review has provided an updated synopsis of the current state of knowledge of drugs and resistance in HCMV (Gilbert and Boivin, 2005).

HIV

The development of antiretroviral drug resistance is currently a significant cause of treatment failures in HIV-infected patients (Hirsch et al., 2000; Hirsch et al., 1998). Genotyping for detection of mutations that confer resistance has become a routine component of management of HIV-infected individuals. Because of the lack of proofreading activity in the RT enzyme that copies the genome of HIV, the virus exists in infected individuals as a population of variants or quasispecies (Delwart et al., 1998). This results in the random appearance and subsequent selection of resistant mutants in the presence of a selection pressure such as an inhibitory drug. Initially, a single mutation may occur, confer a low level of resistance, and then grow to predominance in the population. With ongoing replication, additional mutations that confer high-level resistance appear and the population as a whole becomes highly resistant (Martinez-Picado et al., 1999a). For some drugs, such as lamivudine and nevirapine, a single mutation confers high-level resistance (Shafer, 2002).

Genotypic methods that have been used for detection of mutations in HIV include sequencing, selective PCR, oligonucleotide-specific hybridization, microarray hybridization (GeneChip; Affymetrix, Inc., Santa Clara, CA), and reverse hybridization (line probe assay [LiPA]; Bayer Diagnostics NAD, Norwood, MA). The LiPA for detection of mutations in HIV is currently not commercially available. Sequencing methods have been developed by several noncommercial laboratories and by two commercial companies, Abbott Diagnostics (Abbott Park, IL; formerly available from Applied Biosystems, Inc.) and Bayer Diagnostics (Tarrytown, NY; formerly available from Visible Genetics, Inc.). Shafer (2002) has reviewed the various assays available for genotypic analysis of HIV-1.

Dideoxynucleotide sequencing is the most commonly used method of HIV-1 PR and RT gene sequence analysis (Demeter et al., 1998; Shafer, 2002). Two commercially available systems have been used successfully for this purpose, the TrueGene HIV-1 genotyping kit and OpenGene DNA sequencing system (Siemens Medical Solutions Diagnostics, Tarrytown, NY) and the ViroSeq HIV-1 genotyping system (Celera/Abbott Diagnostics) (Cingolani et al., 2002; Cunningham et al., 2001; Erali et al., 2001; Mracna et al., 2001; Tural et al., 2002). The TrueGene HIV-1 genotyping system has been approved for in vitro diagnostic use. The sequencing systems rely on initial extraction of viral RNA from patient

plasma, RT-PCR of the extracted RNA to amplify about 1,500 bp of PR and RT, and then a sequencing reaction with dye terminators (ViroSeq) or dye-labeled primers (TrueGene). Both companies make sequencing instruments for electrophoresis of the samples, and both provide software to assemble the segments and align the assembled patient sequence with a reference sequence to aid in the identification of mutant codons. With the primers for PCR and the sequencing primers, the ViroSeq system provides double coverage at a minimum for the entire 1.5-kb sequence of interest and quadruple coverage may be obtained for certain regions. The ViroSeq software combines the functions of several previously available software packages to trim the sequence segments, generate a contiguous consensus sequence that is generated by assemblage of the overlapping individual segments, and align the consensus sequence with a reference sequence. The operator must manually toggle through the entire sequence and edit (confirm or override) the computer base calls and also make the final decision about polymorphic sites where a mutant base might constitute a minor proportion in the background of wild-type bases. The ABI software prints a complete mutation report that identifies "reported" and "novel" mutations based on the Los Alamos National Laboratory HIV database, which is available online at http://hiv-web.lanl.gov. The ViroSeq system has been used to successfully detect RT and PR gene mutations in pediatric samples and in non-subtype B HIV-1 samples (Cunningham et al., 2001; Mracna et al., 2001).

The TrueGene genotyping kit may be used in conjunction with a number of extraction procedures. The purified viral RNA is amplified in a single-tube RT-PCR step, the product of which can be used directly in the subsequent sequencing reactions. It employs a proprietary methodology using 4 pairs of sequencing primers. The upstream primer of each pair is labeled with one dye, and the downstream primer of each pair is labeled with another dye. Each primer pair is present in each of 4 dideoxy-terminator reactions, one reaction for each of the four dideoxy bases. Thus, this arrangement requires 16 different reactions and 16 lanes on the sequencing gel to cover the HIV PR and RT genes in both directions. The data are analyzed with the TrueGene software and interpreted to provide a resistance report. Two studies have demonstrated improved virological outcome when genotyping information is considered in patient management decisions (Cingolani et al., 2002; Tural et al., 2002).

A number of studies have compared the various homebrew and commercial sequencing methods. An interlaboratory study compared the ability of 13 laboratories to detect RT mutations in cultured PBMC pellets. Highly concordant results were obtained overall, with some difficulty encountered with a clinical isolate that contained a mixture of wild-type and mutant codons. A mutant codon which was present at less than 50% of a mixed population of resistant and susceptible genotypes was not consistently detected (Demeter et al., 1998). Two large multicenter studies compared sequencing results among participating laboratories for detection of RT mutations in either coded plasmid mixtures or spiked plasma samples. Laboratories had difficulty in detecting mutations where the mutant represented 25% or less of the total DNA population at that codon (Schuurman et al., 2002; Schuurman et al., 1999). Even the editing process can contribute variability to the overall HIV genotype determination as shown by Huang et al. (2003). In this study, sequence concordance was high even though different editing strategies were used by different labs, but 12% of the resistance mutations present in the 10 electronic files that were distributed and analyzed were not identified in some labs.

The LiPA was manufactured by Innogenetics but marketed by Bayer Diagnostics NAD as VERSANT HIV-1 RT resistance assay and VERSANT HIV-1 PR resistance assay. It has been withdrawn from the market.

The Virtual Phenotype assay (Virco) utilizes a proprietary algorithm to compare a virus genotype to a large database of known genotypes and phenotypes to predict a phenotype based on sequence data (Hanna and D'Aquila, 2001; Shafer, 2002). Two RT and PR gene sequence analysis programs are available online from Stanford University at http://hivdb.stanford.edu (accessed 7 August 2007). One program compares an HIV-1 sequence submitted by the investigator to a consensus reference sequence ultimately linking RT and PR gene sequence variations to the antiretroviral therapy history of the patient from whom the sequences were obtained (Shafer et al., 2000). In the second sequence analysis program, Drug Resistance Interpretation, PR and RT gene sequences are entered by the user, and the program produces a phenotypic interpretation based on correlations of genotype and accumulated information on clinical outcome (Shafer, 2002).

Monogram Biosciences, Inc., offers a genotyping assay, GeneSeq HIV, using the dideoxynucleotide chain termination method of DNA sequencing. Based upon the mutations detected, an interpretation algorithm is applied to predict a resistance profile. The PhenoSense GT (Monogram Biosciences) combines the results of the PhenoSense HIV with the GeneSeq HIV, providing a net assessment of drug resistance as well as a listing of mutations detected.

In addition to the databases and interpretation systems discussed previously, the International AIDS Society-USA maintains a database of resistance-associated mutations in the RT, PR, and envelope genes at http://www.iasusa.org/resistance_mutations (accessed 7 August 2007). Other genotype interpretations systems are available commercially or publicly (Van Laethem and Vandamme, 2006). A concern with each of the genotypic methods is that mutant variants present at low frequency may not be detectable and that mixtures of HIV-1 strains with minor sequence variations may not be distinguishable (Hanna and D'Aquila, 2001; Shafer, 2002). Genotypic assays do allow more rapid and efficient detection of resistance than phenotypic assays and may allow earlier detection of emerging resistance than phenotypic assays. Also, because of the complex interactions among different combinations of resistance mutations, predictions of phenotype based on genotype alone may not be accurate (Boyer et al., 1998; Shafer, 2002). Moreover, genotypic assays can only detect known resistance-associated mutations. The complexity of these tests makes them impractical for many diagnostic virology laboratories. Measurement of HIV-1 RNA levels in plasma (viral load) reflects the extent of virus replication in an infected individual and remains the strongest predictor of clinical outcome. Declining HIV-1 RNA levels during treatment indicates response to therapy, while a significant rise in RNA levels indicates treatment failure (Marschner et al., 1998) (http://www.aidsinfo.nih.gov).

None of these genotypic methods is currently able to detect resistance to the newest antiretroviral agent, enfuvirtide (T-20). This is a synthetic peptide inhibitor that interferes with viral entry by blocking formation of a hairpin structure that is necessary for fusion of the viral membrane

with the cell membrane. Resistance to enfuvirtide is conferred rather quickly (within weeks) and is the result of a single mutation or a combination of mutations within the *env* HR1 domain; the substitutions most frequently associated with enfuvirtide resistance occur in residues 36 to 45 (Lu et al., 2006). Sequence analysis of the *env* HR1 domain is required to document enfuvirtide resistance.

Maraviroc is a second entry inhibitor, recently approved for treatment of adult patients with HIV-1 strains resistant to multiple antiretroviral agents, which exploits the requirement for HIV-1 to bind a coreceptor, in addition to the CD4 receptor, for viral entry into the host cell. The two major coreceptors for HIV-1 are CXCR4 and CCR5, expressed on T lymphocytes and macrophages, respectively. Maraviroc is a noncompetitive inhibitor of the chemokine coreceptor, CCR5; it selectively binds to CCR5, preventing fusion between HIV-1 viral and cellular membranes and, thus, entry to cells. Because some HIV-1 strains are also tropic for CXCR4 and some strains are dualtropic using both CXCR4 and CCR5, strains must be characterized, prior to initiation of maraviroc therapy, by means of a tropism assay to determine the identity of the required receptor. A commercially available HIV tropism assay is offered by Monogram Biosciences, Inc. Maraviroc resistance has been selected in vitro and was associated with amino acid substitutions or deletions in the V3-loop region of the HIV-1 envelope glycoprotein; clinical resistance has not yet been characterized.

Of recent concern with respect to the now greater availability of antiretrovirals in resource-limited countries is the question of whether the genotyping methods that have been developed for use in Western countries, mainly on HIV group M subtype B virus, will work on non-B subtypes. This concern is significant, since subtype B accounts for only about 12% of the global AIDS pandemic. In a set of 35 HIV isolates from group M subtypes A to J, full-length sequences were created with the ViroSeq reagents in 84% of the specimens tested and with the TrueGene in 53% of the specimens tested (Beddows et al., 2003). Both methods amplified RNA from plasma levels of about 100 to 1,000 copies/ml. Eshleman et al. (2004) employed the ViroSeq system to genotype 126 samples (114 of which were non-B subtypes) and successfully sequenced 124 of these. Genotypes performed in a second lab on the same 126 samples were 98 to 100% identical to the primary lab results. Thus, it appears that the two commercially available genotyping systems work tolerably well on subtypes other than B and should be generally usable in geographic regions where non-B subtypes predominate.

Aside from the mechanics of performing HIV genotypic assays on non-B subtypes, is the question of whether these subtypes exhibit drug-resistant mutation patterns in the same way and at the same locations as the subtype B strains. In an authoritative recent paper on this topic, Kantor et al. (2005) looked at the correlation of antiretroviral treatment on the distribution of mutations in HIV sequences from 3,686 individuals infected with non-B subtypes and compared the data with mutations in HIV sequences from 4,769 patients with subtype B. All of the known subtype B resistance mutations occurred in the non-B subtypes, and 80% were correlated with antiretroviral treatment of the patients with the non-B subtypes. The authors concluded that it is reasonable for global monitoring of resistance to continue to focus on the known subtype B resistance mutations, as there are apparently no unique or previously unrecognized mutations in non-B subtypes.

HBV

Treatment of chronic HBV infections with antiviral drugs and immune modulators is becoming more widely employed, as recent clinical trials have demonstrated efficacy. Four nucleoside analogs, lamivudine, adefovir dipivoxil, telbivudine, and entecavir, a carbocyclic deoxyguanosine analog (Shaw and Locarnini, 2004), are approved for treatment of chronic HBV and act at the level of the HBV DNA polymerase. Alpha interferon (IFN-α) and pegylated IFN-α (PegIFN) are approved for treatment and have both antiviral and immunomodulating activity. However, PegIFN has replaced IFN-α, due to its more convenient dosing schedule. Long-term treatment regimens suppress HBV replication but do not eradicate the virus; discontinuation of therapy results in rebound of HBV replication unless HBV e antigen (HBeAg) seroconversion is achieved.

Three regions of the HBV genome are relevant to treatment, resistance to the various drugs, and type/subtype determination: (i) the active site of the viral DNA polymerase gene, (ii) mutations in the precore (preC) region of the HBV genome, and (iii) mutations in the core (C gene) and the surface (S gene) proteins.

Lamivudine was the first clinically useful drug for treatment of HBV and is still widely used today, although resistance is virtually assured (approximately 20% and 70% of patients develop resistance after 1 and 5 years of lamivudine therapy, respectively) if the drug is used long enough in any given patient (Lok and McMahon, 2007). The active site of the HBV DNA polymerase enzyme is very similar to that of HIV (they both have the same complement of enzymatic activities), and thus, it seemed reasonable that many of the nucleoside analogs that inhibit HIV RT would also inhibit HBV polymerase. The most common locus of lamivudine resistance mutations, rtM204V/I/S, is located within the YMDD motif in the C domain of the viral RT gene (the "M" of the YMDD motif). The rtM204I mutation conferring lamivudine resistance can occur independently of other mutations; however, rtM204V/S are found only in conjunction with other mutations such as rtL180M/C (Shaw et al., 2006). rtL180M/C mutations, located in domain B of the RT, are insufficient alone to confer resistance but enhance levels of lamivudine resistance and improve the replication fitness of rtM204I/V/S (Shaw et al., 2006). Other rarer mutations are rtL80V/I, rtI169T, rtV173L, rtA181T, rtT184S, and rtQ215S, many of which are compensatory mutations, i.e., they enhance viral fitness of lamivudine-resistant mutants (Shaw et al., 2006; Valsamakis, 2007; Yuan and Lee, 2007). Adefovir dipivoxil, an acyclic analog of dAMP, suppresses HBV replication at a slower rate than lamivudine or entecavir but selects for resistant mutations at a much slower rate than lamivudine (Shaw et al., 2006; Yuan and Lee, 2007). However, adefovir-resistant mutants emerge more rapidly in patients with lamivudine-resistant HBV. In addition to primary therapy for chronic HBV, adefovir also has been used as rescue therapy for treatment of lamivudine-resistant HBV. Mutations associated with adefovir resistance in vivo are rtN236T and rtA181V/T. Other, less frequently occurring, mutations associated with adefovir resistance are L80V/I, V84M, V214A, S85A, Q215S, P237H, and N238T/D (Angus et al., 2003; Arens, 2001; Shaw et al., 2006; Yuan and Lee, 2007).

Entecavir was approved by the U.S. Food and Drug Administration (FDA) in 2005 for treatment of chronic HBV. This development is particularly exciting because entecavir is active against lamivudine- and adefovir dipivoxil-resistant

HBV; it is easily absorbed, well tolerated, and relatively nontoxic. Entecavir is a carbocyclic deoxyguanosine analog that acts directly on the HBV DNA polymerase to cause delayed or nonobligate chain termination and also blocks priming of the RT reaction (Shaw and Locarnini, 2004). Resistance to entecavir is rare, having occurred in only two patients of the more than 500 enrolled in phase II and III clinical trials (Tenney et al., 2004). Interestingly, resistance to entecavir alone was not observed; the two patients with resistance to entecavir had preexisting mutations to lamivudine (rtL180M/rtM204V) which, in combination with new entecavir-induced mutations (rtM250V/rtI169T and rtT184G/rtS202I), resulted in high-level resistance to both drugs (Shaw et al., 2006; Tenney et al., 2004). Thus, patients with lamivudine resistance due to mutations at codons 180 and 204, when exposed to entecavir, may develop additional RT mutations and be highly resistant to both drugs (Tenney et al., 2004). A fourth nucleoside analog, telbivudine, approved by the FDA in 2006 for treatment of chronic HBV infection, inhibits HBV DNA polymerase, causing DNA chain termination and resulting in inhibition of HBV replication. Telbivudine resistance was associated with the M204I mutation (Lai et al., 2005). Lamivudine-resistant strains with mutations in the YMDD motif are cross resistant to telbivudine (Yuan and Lee, 2007). Strategies for rescue therapy of patients with antiviral-resistant HBV must take into account prior treatment received, pattern of mutations found, and cross-resistance with alternative agents (Lok, 2007).

As with HIV-1, both genotypic and phenotypic assays have been utilized to detect HBV drug resistance mutations (Shaw et al., 2006). Sequence analysis of the active site of the HBV polymerase is most commonly used for detection of the mutations known to confer resistance to these drugs. Direct sequencing is problematic because it is unable to detect resistant mutants in low concentrations. Cloning of the HBV polymerase region followed by sequence analysis of inserts from single recombinant clones is more sensitive but not amenable to clinical laboratories (Shaw et al., 2006). Two commercial direct sequencing assays, TRUGENE HBV genotyping kit (Siemens Medical Solutions Diagnostics) and Affigene HBV DE/3TC assay (Sangtec Molecular Diagnostics AB, Bromma, Sweden) amplify and sequence either a 1.2-kb sequence of the HBV RT gene, encompassing the central portion of the RT domain (TRUGENE HBV), or codons 180 and 204 (Affigene) (Olivero et al., 2006; Woo et al., 2007). Restriction fragment length polymorphism analysis can detect mutants at a level as low as 5%, but again, it is not amenable to a clinical laboratory (Shaw et al., 2006). Various DNA hybridization assays have been described previously (Shaw et al., 2006), including a second-generation, commercial, LiPA, INNO-LiPA HBV DR v2 (Innogenetics N.V., Ghent, Belgium), which detects lamivudine resistance mutations at codons 80, 173, 180, and 204 and adefovir resistance mutations at codons 181 and 236 of HBV polymerase (Hassain et al., 2006; Osiowy et al., 2006). Advantages and disadvantages of various genotypic assays for HBV drug resistance have been reviewed by Valsamakis (2007).

In addition to genotypic assays, phenotypic assays have been utilized for characterization of HBV drug resistance. A variety of phenotyping methods have been employed and are reviewed by Shaw et al. (2006). One method involves generation of point mutations associated with drug resistance by site-directed mutagenesis of laboratory strains of HBV followed by transfection of permissive cell lines with plasmid vectors containing the mutation of interest and subsequent exposure to antiviral drug(s). The phenotype is deduced by comparing replication of cell lines with or without mutations in the presence of drug. Similarly, full-length HBV genomes also have been used for transfection experiments. Another strategy is the use of recombinant baculoviruses to deliver HBV genomes into cell culture systems, such as HepG2 cells, which are then exposed to antiviral agents. Alternatively, virtual phenotyping correlates patient clinical data and viral mutational data to assign an antiviral drug phenotype (Shaw et al., 2006). SeqHepB is a combination of an HBV genome sequence analysis program and a relational database containing data from multiple sources (Yuen et al., 2007). The program determines genotype and performs mutational analysis of HBV genomic sequences to generate a resistance profile based on the primary and secondary mutations found in the sequence. The sequence analysis component of SeqHepB (Last Resort Support Pty Ltd., Victoria, Australia) can be accessed by registered users with their username and password via the Internet.

The preC region of the HBV genome has recently gained significance because there are indications that it may be associated with IFN resistance. There is long-standing evidence (Fattovich et al., 1995) that mutations in the preC region have a negative effect on IFN treatment, and more-recent in vitro experiments have supported this contention (Wang et al., 2005). The development of a G1896A (codon 28) mutation results in a termination codon that interrupts translation of the HBeAg precursor and thus initiates an e-antigen-negative chronic hepatitis. Sequencing of this region is a reasonable method for detection of these mutations. Alternatively, Innogenetics has a LiPA (INNO-LiPA HBV PreCore) that is capable of detecting this mutation as well as preC promoter mutations that may also be present (codons 1762 and 1764). Furthermore, classification of HBV into types A to H, based on the genomic sequence, has yielded unexpected correlations with the clinical course of disease. It has long been known that some types, for example, type B, are associated with less-active liver disease, slower progression, and more likely spontaneous HBeAg seroconversion than other types, for example, type C (Orito et al., 2001). Recent studies with PegIFN (Janssen et al., 2005) have yielded the clinically relevant information that the response was type dependent. In this study, patients with type A had a 47% response (i.e., loss of HBeAg), patients with type B had a 44% response, patients with type C had a 28% response, and patients with type D had a 25% response. Thus, there is increasing evidence that determination of the HBV type is an important predictive factor in IFN treatment and the overall course of disease (Fung and Lok, 2004). The genotype can be determined by sequencing the entire genome, the core region, or the surface protein region. Two commercially available assays, INNO-LiPA HBV genotyping kit (Innogenetics) and TRUGENE HBV genotyping kit (Siemens), identify HBV genotype based on type-specific sequences in the HBV S gene region (Gintowt et al., 2005; Osiowy and Giles, 2003).

Current recommendations for monitoring of patients on therapy for HBV include observing baseline quantitative HBV DNA levels and additional levels periodically to help determine the endpoint of treatment in patients with chronic hepatitis B (American Association for the Study of Liver Diseases) (Lok and McMahon, 2007). Genotyping for detection of resistance should be performed if resistance is suspected because of rising DNA levels or appearance of symptoms (Lok, 2007; Lok and McMahon, 2007).

INTERPRETATION OF ANTIVIRAL SUSCEPTIBILITY RESULTS

Table 1 lists breakpoint IC_{50}s proposed by various investigators for herpes group viruses and influenza viruses. The concentration of antiviral agent by which virus is considered susceptible has generally been based on median susceptibilities of large numbers of clinical isolates from patients prior to, during, and after antiviral therapy. Because of the variables that affect antiviral susceptibility results, the absolute IC_{50} can vary from assay to assay and from laboratory to laboratory. Moreover, in vitro results indicating susceptibility or resistance may not correlate with response of the infection to therapy in vivo. The clinical response of the patient depends upon a number of other factors, such as immunological status and pharmacokinetics of the drug in that particular patient (dose or route of administration could be inappropriate). A poor clinical response may occur even though the antiviral susceptibility testing denotes in vitro susceptibility (Dekker et al., 1983). Patients with HSV infections who are immunocompromised may fail to respond to therapy despite in vitro IC_{50}s indicating susceptibility to vidarabine or acyclovir (Englund et al., 1990; Safrin et al., 1990; Safrin et al., 1991b). Conversely, HSV isolates for which IC_{50}s of acyclovir are >2 μg/ml can occasionally be recovered from otherwise healthy hosts who have responded to acyclovir therapy (Lehrman et al; 1986). Thus, a high IC_{50} derived by in vitro susceptibility testing is not sufficient to designate a viral strain as resistant, nor can in vitro susceptibility to a drug a priori predict successful clinical outcome. Whenever possible, evidence of genetic alteration of the virus should be considered as well.

Interpretation of antiviral susceptibility results is further complicated by the variability in endpoint due to testing methodologies (Cole and Balfour, 1987; Hill et al., 1991a; McLaren et al., 1983; Pepin et al., 1992). Because endpoints are dependent on test method, each new method and antiviral agent must be correlated with a historic standard that has been used to test large numbers of isolates. Also, the absolute IC_{50} may vary from assay to assay and laboratory to laboratory. Moreover, because small subpopulations of resistant virus may not be reflected in IC_{50}s, IC_{90}s may be more predictive of clinical response. One approach to interpreting susceptibility endpoints is to compare the IC_{50}s of an isolate obtained prior to therapy (or of a well-characterized reference control strain) with that of an isolate obtained during therapy; a significant increase in the ratio of such IC_{50}s denotes resistance. However, pretherapy isolates are often unavailable, and the IC_{50} ratio considered clinically significant is unclear. Large-scale collaborative comparisons of methods with the same viral isolates are necessary to standardize antiviral susceptibility testing and to establish definitive interpretive guidelines. Only when a standardized assay is adopted can prospective studies be performed to correlate in vivo response with in vitro susceptibility. Such studies are essential before definitive interpretive breakpoints are established.

Susceptibility testing of penciclovir illustrates the effect that cell line and testing method have on endpoint. When acyclovir and penciclovir, which is structurally similar to acyclovir, were tested with HSV isolates in Vero cells by PRA, penciclovir appeared less active than acyclovir and HSV type 2 isolates appeared to be resistant to penciclovir. In contrast, penciclovir appeared more active than acyclovir against some HSV isolates when SCC25 cells were used. Both drugs appeared to have comparable activity when tested in MRC-5 and A549 cells (Leary et al., 2002). When clinical isolates of VZV were tested by plaque reduction and DNA hybridization, IC_{50}s for DNA hybridization were significantly lower than those by PRA (Standring-Cox et al., 1996). Variability in endpoint also was seen with VZV depending on the composition of the inoculum (cell free versus cell associated). Therefore, breakpoints for susceptibility testing of penciclovir with HSV and VZV cannot be established at this time (Leary et al., 2002; Standring-Cox et al., 1996).

A limitation of phenotypic testing for HIV-1 is the lack of consensus as to the absolute IC_{50}s denoting resistance of HIV-1 to antiretroviral drugs. In vitro susceptibility of HIV-1 results are usually expressed as the relative (*n*-fold) increase in the IC_{50} of an isolate obtained on therapy compared to a pretreatment isolate or a drug-susceptible isolate (Hertogs et al., 1998; Schinazi et al., 1997). The increase in the IC_{50} considered clinically significant is likely to vary by drug or drug class (Hanna and D'Aquila, 2001). The Antivirogram assay (Virco) has established cutoffs denoting reduced susceptibility at 2 standard deviations above the mean value for 1,000 isolates from untreated HIV-positive individuals and for several thousand isolates of genetically wild-type virus. Both Virco and ViroLogic are aware of the importance of clinical cutoff values and are making diligent efforts to ascribe accurate values for use by physicians.

The significance and clinical utility of phenotypic and genotypic analysis of HIV-1 is increasingly being recognized. A report that reanalyzed data from previous studies demonstrated that baseline genotypic and phenotypic drug resistance predicted virological failure (DeGruttola et al., 2002). Genotypic analysis of the PR gene in patients on antiretroviral therapy but PI-naive has also been shown to be predictive of virological and immune response to therapy with PIs (Perez et al., 2001). Prospective studies have demonstrated improved virological outcome when genotyping information is considered in patient management decisions (Cingolani et al., 2002; Durant et al., 1999; Tural et al., 2002). Drug resistance testing is now recommended to help guide selection of salvage therapy after treatment failure and for guiding therapy for pregnant women (Hirsch et al., 2000). However, limitations of genotypic testing include the difficulty in interpretation of resistance mutations and inability to detect minority variants.

It is important to remember that antiretroviral therapy may fail for reasons other than the emergence of drug-resistant virus, such as drug antagonism, nonadherence, increased clearance of one antiretroviral drug when coadministered with another drug, inadequate penetration of drug into a sequestered site, i.e., central nervous system, or malabsorption of drug from the gastrointestinal tract (Hanna and D'Aquila, 2001; Hertogs et al., 1998).

FUTURE DIRECTIONS

Consensus protocols for antiviral susceptibility testing are under development. A protocol for HSV plaque assays is now published (NCCLS, 2004), and a standardized procedure for sequencing of the HCMV UL97 gene (Lurain et al., 2001) has been published. Standardization is hampered by the many variables that affect susceptibility testing results. No single assay method, cell line, or inoculum composition (cell free versus cell associated) appears sufficient for testing all viruses. A major problem with culture-based susceptibility testing of viruses other than HSV is that assays may require weeks to complete, a fact that limits their utility in management of acute cases. Genotypic assays for UL97 and

UL54 mutations of HCMV have largely replaced culture-based assays as more resistance-associated mutations have been identified. Detection of antiviral resistance in patients with HIV-1 and HBV infections is considered an integral component of patient management and will continue to evolve as newer antiviral agents are approved.

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Application of Western Blotting to Diagnosis of Viral Infections

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Molecular biological techniques have an increasing role in the laboratory diagnosis of viral infections as a consequence of their being more sensitive and specific than methods developed in the past. These modern techniques are especially important for the diagnosis of infections caused by agents that are difficult to propagate in tissue or cell culture. For example, techniques such as Western blotting (also referred to as immunoblotting) and PCR are part of the growing panel of diagnostic procedures for human immunodeficiency virus (HIV) and human T-cell leukemia virus type 1 (HTLV-l) and HTLV-2 infections (Gallo et al., 1986; Mullis et al., 1986; Carlson et al., 1987, Centers for Disease Control, 1988a, 1988b, 1989; Saiki et al., 1988; Consortium for Retrovirus Serology Standardization, 1988; Healey and Howard, 1989; Hirsch and Curran, 1990). This chapter discusses the principle, describes the methodology, and provides some practical clinical applications of Western blotting.

HISTORY AND PRINCIPLE OF WESTERN BLOTTING

The diagnosis of viral infection is often based on the detection of specific circulating antibodies to viral antigens in serum samples. Enzyme immunoassays (EIA) often are used for diagnosis of viral diseases as well as for screening of blood and blood products for viruses. Although EIA are very sensitive and highly specific, false-positive reactions occur. Given the medical and social significance of particular virus infections, e.g., HIV, HTLV, and the hepatitis viruses, it is important that diagnostic tests for these virus infections be as specific, accurate, and sensitive as possible. Although the sensitivities and specificities of some EIA can be greater than 95%, e.g., the licensed EIA for HIV (Petricciani, 1985; Centers for Disease Control, 1988b), it is a standard laboratory practice to repeat a positive EIA. According to guidelines adopted by the U.S. Public Health Service, if a second positive EIA result is obtained upon repeat testing, the diagnosis must be confirmed by another assay, most often Western blotting (Centers for Disease Control, 1988a, 1988b, 1989; Consortium for Retrovirus Serology Standardization, 1988).

Recently, the FDA approved several new rapid HIV tests for use as more accessible point-of-care tests. These highly sensitive tests are taking the place of the traditional EIA because they do not require specialized laboratory equipment and are less invasive, requiring oral fluid, urine, or finger-stick blood rather than serum drawn by venipuncture. This increase in accessibility and efficiency means that rapid tests can be used in most health care settings and provide results the same day without sacrificing sensitivity. The Centers for Disease Control and Prevention has described protocols for confirming these new tests, emphasizing that all reactive rapid HIV test results must be confirmed by either the more specific Western blot or immunofluorescent assay, even in the event that a subsequent EIA is nonreactive, and that negative or indeterminate confirmatory tests should be repeated 4 weeks after the initial reactive rapid test result (Centers for Disease Control and Prevention, 2004). These confirmatory tests must be done by trained personnel at laboratories equipped to perform more complex tests.

Several blotting techniques have been developed. The initially described technique is referred to as Southern blotting, after its originator, E. M. Southern (1975). It is a fundamental tool for the analysis of DNA fragments. An analogous method for analyzing RNAs was dubbed Northern blotting (Alwine et al., 1977) as a molecular biologist's joke. The humor continued when a modification of the nucleic acid blotting methods for the study of proteins was developed and referred to as Western blotting, more properly called immunoblotting (Towbin et al., 1979; Burnette, 1981).

These different blotting techniques share a common principle. Complex mixtures of macromolecules (DNA, RNA, or protein) are first separated by size in rectangular slab gels by using electrophoresis. After separation, the molecules are transferred (blotted) onto the surface of a membrane, and the separated and immobilized nucleic acid fragments or proteins are detected and/or identified on the membrane by using specific molecular probes. For example, RNA blots can be probed with radioactive cDNA probes. The bound RNA hybridizes in situ with the labeled probe, and the reaction is then visualized by autoradiography (Alwine et al., 1977). The basic approach to the use of Western blotting for the diagnosis of viral infections begins with purified virions that are disrupted by ionic detergent treatment, releasing viral proteins. As shown schematically

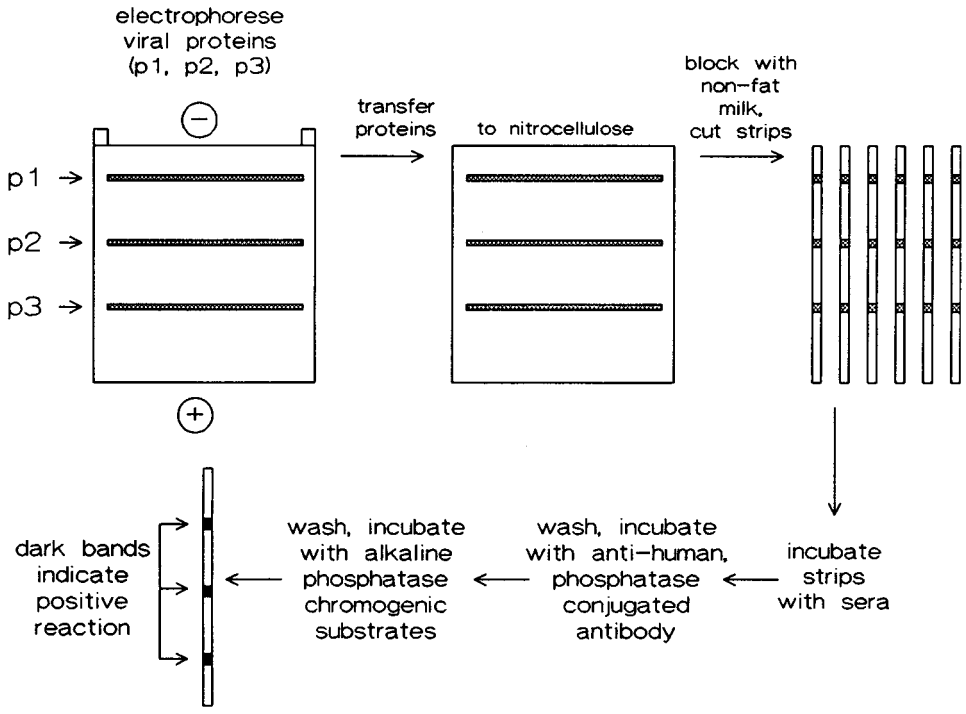

FIGURE 1 Schematic representation of Western blotting.

in Fig. 1, these virion proteins are then separated on gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). These gels, approximately 1-mm thick, are formed between two square glass plates; 14 by 14 cm is a typical size. Viral proteins are denatured by boiling in SDS–2-mercaptoethanol buffer, and a sample containing a few hundred micrograms of protein is loaded across the top of the gel. After electrophoresis, the gel is placed on a membrane (nitrocellulose or nylon, etc.) of the same size, and this gel-nitrocellulose unit is placed on top of several layers of wet filter paper. Filter papers are then laid on the gel and proteins are transferred (blotted) to the membrane by electrophoresis. Transfer may be done by semidry electroblotters when the gel-nitrocellulose-paper sandwich is placed between two rectangular metal or carbon electrodes (apparatus available from Hoefer Scientific Instruments Inc., San Francisco, CA; Fisher Inc., Springfield, NJ; or other distributors and manufacturers). In an older version of electroblotting, which is still preferred by some investigators, the sandwich is held together by a plastic device and is submerged in buffer in a large electrophoresis tank (Towbin et al., 1979; Burnette, 1981). In either case, the transfer of proteins from the gel to the membrane is mediated by electrical current so that the pattern of proteins obtained by SDS-PAGE is preserved during transfer. After transfer, the membrane is incubated with a buffer containing nonspecific proteins such as milk casein or serum albumin to block all unoccupied areas that could serve as binding sites. This blocking step prevents nonspecific adsorption of immunoglobulin (Ig) proteins to the nitrocellulose filter during subsequent steps. The nitrocellulose sheet is then cut into several strips, and each strip is ready to use for the detection of antiviral antibodies.

If a patient serum containing anti-HIV antibodies is incubated with a Western blot strip, the antibodies specific to the individual viral proteins form stable complexes with the transferred protein species, and the antibodies remain bound to those antigens even after extensive washing. Typically, patients who have seroconverted have antibodies to several proteins of the viral agent (Centers for Disease Control, 1988a, 1988b, 1989; Consortium for Retrovirus Serology Standardization, 1988).

After washing to remove unbound antibodies, the last step in the procedure is to visualize the patient's bound Ig. This can be achieved by using radiolabeled *Staphylococcus aureus* protein A or a second antibody that is labeled with a radioisotope or an enzyme. Clinical laboratories usually use safer, nonradioactive methods such as alkaline phosphatase or horseradish peroxidase enzyme-coupled anti-human antibodies. The anti-human Ig and the enzyme are coupled covalently in a manner that allows the activity of both molecules to remain intact.

Detection of patient antibody is accomplished by monitoring enzyme activity linked to the anti-human antibody (see chapter 9, this volume). The enzyme activity can be demonstrated in situ by incubating the membrane with appropriate chromogenic or luminogenic substrates. The products of chromogenic reactions are insoluble in water and develop a dark color at the site of enzyme activity. During the last step of the Western blot procedure, dark bands (blue for alkaline phosphatase, brown for peroxidase) corresponding to the physical location of the reactive viral proteins appear on the nitrocellulose filter. Luminogenic substrates produce light at the site of enzyme activity. Luminol releases blue-green light when it is oxidized by a reaction involving hydrogen peroxide and horseradish peroxidase. The reaction is enhanced in the presence of phenols, which increase light output and duration. A permanent record of results is obtained by exposing membranes to blue light-sensitive X-ray film a few

minutes after addition of substrates. Film is exposed for up to 1 h, although exposures from 10 s to 10 min are usually all that are necessary.

In summary, the cascade of steps in performing Western blotting includes electrophoretic separation of viral proteins, nitrocellulose immobilization of viral proteins, viral protein capture of patient antibodies, patient antibody binding to enzyme-labeled anti-human antibody, and detection of the presence of human antibody by an enzyme reaction that produces visible dark bands or chemiluminescence at the site of these molecular complexes.

THE WESTERN BLOT PROCEDURE

The various aspects of purification of virions and viral proteins and the details of SDS-PAGE are not discussed here, since clinical laboratories rarely have sufficient resources to perform these steps. We recommend the purchase of blotted viral proteins or Western blot kits available from several commercial sources.

List of Materials Needed

Instruments and Materials

Rocking platform

Vacuum aspirator with flasks

pH meter

Adjustable micropipettes (1 to 20 μl, 10 to 200 μl, and 100 to 1,000 μl) and sterile tips

Pasteur and serological pipettes

Western blot incubation trays

Nitrocellulose strips—usually shipped in Western blot trays. If you wish to purchase separate trays, two types are available: disposable and reusable. Reusable trays require thorough cleaning after exposure to reagents; disposable trays are more convenient but more costly in the long run. Before ordering trays, contact the manufacturer of the Western blot nitrocellulose strips you plan to purchase, since dimensions of the strips and trays vary among different commercial sources.

Digital or other camera to photograph results for permanent record

Reagents

Tris base (Trizma [Sigma product T-8524 or equivalent]; Sigma Chemical Co., St. Louis, MO)

Hydrochloric acid (5 N)

Sodium chloride (Sigma product S9625 or equivalent)

Magnesium chloride, $6H_2O$ (Sigma product M9272 or equivalent)

EDTA (Sigma product E5134 or equivalent)

Milli-Q or equivalent highly purified or double-distilled H_2O, autoclaved

Nonfat dry milk (from any supermarket)

Nitrocellulose strips with blotted proteins (see below)

Anti-human IgG, alkaline phosphatase conjugated (product S3821 or equivalent; Promega, Madison, WI)

Tween 20 (polyoxyethylenesorbitan monolaurate [Sigma product P1379 or equivalent]) Nitroblue tetrazolium (NBT) powder or NBT tablets (Sigma product N 6876 or N 5514 [for tablets] or equivalent)

N,*N*-Dimethylformamide (Sigma product D8654)

5-Bromo-4-chloro-3-indolyl phosphate (BCIP), *p*-toluidine salt or BCIP tablets (Sigma product B 8503 or B 0274 [for tablets] or equivalent)

Solutions

TST buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) (1 liter)

Dissolve 1.21 g of Tris base and 8.76 g of NaCl in about 900 ml of sterile Milli-Q H_2O, and adjust pH to 8.0 with 5 N HCl. Add 0.5 ml of Tween 20 and fill with H_2O to 1 liter. Store at 4°C.

TST buffer with 5% dry milk (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) (50 ml)

Dissolve 2.5 g of nonfat dry milk in 50 ml of TST buffer; store at –20°C.

Alkaline phosphatase buffer (l00 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl [pH 9.5]) (l00 ml)

Dissolve 1.21 g of Tris base, 0.10 g of $MgCl_2 \cdot 6H_2O$, and 0.58 g of NaCl in about 90 ml of sterile Milli-Q H_2O, and adjust pH to 9.5 with 5 N HCl. Fill with H_2O to 100 ml. Store at 4°C.

NBT solution (2 ml)

Dissolve 100 mg of NBT powder or tablets in 2 ml of 70% *N*,*N*-dimethylformamide (mixture of 0.7 ml of dimethylformamide and 0.3 ml of H_2O). Store in the dark at –20°C.

BCIP solution (1 ml)

Dissolve 50 mg of BCIP in 1 ml of 100% *N*,*N*-dimethylformamide. Store in the dark at –20°C.

Stop solution (5 mM EDTA, 50 mM Tris-HCl [pH 7.5]) (100 ml)

Dissolve 1.21 g of Tris base, 0.186 g of EDTA, and 0.58 g of NaCl in about 90 ml of sterile Milli-Q H_2O, and adjust pH to 7.5 with 5 N HCl. Fill with H_2O to 100 ml. Store at 4°C.

Step-by-Step Procedure

This description is for a procedure that uses an alkaline phosphatase-labeled second antibody for detection. Some minor modifications may be needed if another system is used (Sambrook et al., 1989).

1. Check condition of equipment required, collect reagents, check expiration date of Western blot strips and sera (do not use beyond expiration date), and prepare solutions.
2. Incubate with primary antibody.
   a. Dilute serum 1:50 in TST buffer–5% dry milk. Prepare enough to allow use of 0.05 ml per cm^2 of Western blot filter strip. For example, if the area of the test strip is 5 cm^2, then a minimum of 0.25 ml of diluted test serum should be prepared. Appropriate controls are essential in Western blotting; positive and a negative control sera appropriately diluted must be included in all assays (manufacturers of Western blot kits offer such controls).
   b. Place Western blot strips in a multiwell tray using forceps, and mark each well for future identification of each reaction.
   c. Tilt tray at about a 30° angle and add 0.05 ml of TST buffer–5% dry milk per cm^2 of filter strip to the bottom of each well. Slowly lower the tray to a horizontal position so that the strips adsorb buffer

gradually and evenly. Add diluted serum to the appropriate wells, close the lid, and incubate at room temperature for 2 h on a rocking platform with gentle agitation.

3. Wash filters five times with TST.
   a. Remove lid, tilt tray, and aspirate the liquid by Pasteur pipette connected to a vacuum flask. Do not allow filters to dry out!
   b. Fill wells about halfway with TST and wash with gentle agitation (rocking) for 7 min.
   c. Remove TST and repeat this wash procedure four times for a total of five washes. Make sure that the buffer is completely removed after each wash.
4. Incubation with secondary antibody.
   a. Dilute the anti-human IgG alkaline phosphatase conjugate according to the manufacturer's specifications in TST buffer–5% dry milk.
   b. Add the diluted serum to each well (0.1 ml of diluted serum per cm^2 of filter strip).
   c. Close the lid and incubate at room temperature for 1 h on a rocking platform with gentle agitation.
5. Wash filters five times with TST, as described in step 3.
6. Incubate with chromogenic substrates.
   During the final period, prepare the chromogenic substrates. Mix 66 µl of NBT stock with 10 ml of alkaline phosphatase buffer in a test tube, and then add 33 µl of BCIP and mix well. Remove the washing buffer and add 0.1 ml of chromogenic substrate solution per cm^2 of filter strip. Incubate the tray with gentle agitation as before and monitor the development of dark blue bands.
7. Stop reaction.
   When strong bands are visible with the positive control serum and test samples but the negative control is still clear, the reaction has stopped. Optimal incubation time with substrates varies from a few minutes to half an hour and depends on the reagents and the titer of antibodies. To stop the reaction, quickly aspirate the substrate solution and add 2 ml of stop solution. Some experimentation is necessary to determine optimal conditions for each new set of reagents and to stop the reaction at the right moment. A timer should be used to monitor the incubation time with the chromogenic substrates to note the optimal time for future reference. Overincubation usually results in high background and/or appearance of bands in the negative control serum. If this should occur, the Western blot must be repeated with a shortened incubation period with the chromogenic substrate.
8. Interpretation of the assay, with special hints.
   In general, a Western blot test is considered positive when the patient's serum is reactive with more than one viral antigen. For example, a licensed HIV test is interpreted as positive when multiple bands are present, e.g., p24, p31, gp41, and gp160 (Centers for Disease Control, l988a, 1988b, 1989; Consortium for Retrovirus Serology Standardization, 1988). The question is how to identify these proteins. Most manufacturers offer sequentially numbered Western blot strips that have originated from a single gel. The strips are numbered such that adjacent numbers refer to originally adjacent strips. The key to accurate results is to use a pair of adjacent nitrocellulose Western blot strips that originated from the same area of the gel used in SDS-PAGE. One of these strips should be reacted with the positive control serum, and the adjacent strip should be reacted with the patient sample. When the test is complete, strips should be aligned numerically according to the manufacturer's numbering and should be compared side-by-side and photographed. This method of analysis can help in the interpretation of dubious and nonspecific reactions.

Alternative Procedure: Chemiluminescence

Chemiluminescence has two main advantages compared to chromogenic techniques. First, it enables a >10-fold increase in sensitivity without the use of isotopes (Schneppenheim et al., 1991; Constantine et al., 1994), and second, exposure times can be varied to increase or decrease sensitivity. A disadvantage is that it requires the use of a darkroom and developing equipment. Although alkaline phosphatase can be used in this technique, systems based on this enzyme are more complex and less convenient than those based on peroxidase. For these reasons, the luminol peroxidase system is recommended. This procedure is identical to that described in the Western blot step-by-step section, with a few exceptions. The secondary antibody used in step 4 must be conjugated to horseradish peroxidase, and steps 6 and 7 should be replaced with the following (Schneppenheim et al., 1991).

1. Add just enough visualization solution to cover each strip, and allow the reaction to proceed for 1 min at room temperature. Remove excess solution from the strips and place them face down on a sheet of clear plastic wrap, and fold the plastic wrap around the strips so that they are completely covered on both sides. Next, place the strips in plastic wrap face up in an X-ray film cassette and make sure the plastic wrap is dry and smooth (wrinkles in plastic wrap between film and strips create high background). Close the cassette until the film is exposed in a darkroom. The film can be exposed immediately; do not wait longer than 20 min, as luminescence will begin to decline.

2. In a darkroom, place the film on top of the strips and close the cassette. The film should first be exposed for only 10 s. If more sensitivity is required, the exposure can be as long as 1 h; longer exposures, however, increase background. If less sensitivity is required, leave the strips in the cassette for an hour or more and repeat exposure.

Materials and Reagents

Visualization solution (prepare immediately before use) (Schneppenheim et al., 1991)

0.5 ml of 101 luminol stock (40 mg of luminol [Sigma product A 8511] in 10 ml of dimethyl sulfoxide). Store at –20°C.

0.5 ml of 101 *p*-iodophenol stock (10 mg [Aldrich product 1-1,020-1] in 10 ml of dimethyl sulfoxide). Store at –20°C.

2.5 ml of 100 mM Tris-Cl, pH 7.5

25 µl of 3% H_2O_2

H_2O (to 5 ml)

Anti-human IgG, horseradish peroxidase conjugated (Promega product W4031 or equivalent)

Clear plastic wrap

Blue light-sensitive X-ray film and cassette

Darkroom and X-ray film developing equipment

ADVANTAGES AND DISADVANTAGES OF THE WESTERN BLOT ASSAY

It is obvious that the Western blot assay is more specific than EIA or rapid tests, since in the Western blot assay, antibodies to several antigens are detected simultaneously by using a group of electrophoretically separated viral proteins. At present, Western blotting offers a very reliable confirmatory assay for HIV-1 infection (Centers for Disease Control, 1988b, 1989; Centers for Disease Control and Prevention, 2004). A potential competitor for the Western blot assay has been reported for the diagnosis of HIV infection. This new technique, called "recombinant-antigen immunoblot assay" (RIBA-HIV216), utilizes a set of purified antigens produced by recombinant technology (Oroszlan and Copeland, 1985; Steimer et al., 1986; Truett et al., 1989; Lillehoj et al., 1990; Busch et al., 1991). It remains to be determined whether this recombinant-protein assay is more specific and/or more sensitive than the standard Western blot assay.

Western blot assays can produce nonspecific, or so-called "indeterminate," reactions in which often only one band is seen. These results are sometimes attributed to, among other causes, an underlying autoimmune disease that results in production of antibodies to cellular antigens (Healey and Howard, 1989). However, indeterminate status may precede a truly positive status and thus be indicative of infection. Such a result requires a specimen to be collected from the patient at a later date for retesting.

Similarly, false-negative reactions have been described (Kissler et al., 1987), but the occurrence of false-negative reactions is relatively rare. Perhaps the main disadvantage of Western blotting is that it requires experienced personnel, which limits its use to laboratories capable of performing more-specialized services. Therefore, training of laboratory personnel to perform this technique is highly recommended.

COMMERCIAL KITS AND NITROCELLULOSE STRIPS WITH BLOTTED PROTEINS

Nitrocellulose strips with blotted viral proteins or complete kits with all necessary reagents are available from commercial sources for HIV-l, HTLV-1, and hepatitis C virus Western blot assays. A current list of all licensed kits and manufacturers can be found at the website of the U.S. Food and Drug Administration (www.fda.gov/cber/products/testkits.htm). Chemiluminescence kits containing premixed visualization solutions and secondary antibody are available from Pierce (Rockford, IL), Amersham Pharmacia Biotech (Piscataway, NJ), and Jackson ImmunoResearch Laboratories (West Grove, PA).

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Nucleic Acid Amplification and Detection Methods

DANNY L. WIEDBRAUK

14

Nucleic acid detection methods play an increasingly important role in the detection of viral infection. Once the province of esoteric and university research laboratories, nucleic acid methods are now an important and necessary part of many hospital laboratories. The rapid evolution of nucleic acid detection technologies and products means that clinical laboratories must choose products and services from an ever-increasing array of vendors and technologies. Each technology has its own testing characteristics, equipment and sample requirements, and sensitivity (Table 1). Matching test performance characteristics and diagnostic utility for an individual institution can be difficult. This chapter will attempt to describe the major nucleic acid testing methods and assist in test selection.

NUCLEIC ACID AMPLIFICATION

Nucleic acid amplification methods are classified as target or probe amplification methods based upon the source of the nucleic acid that is amplified in the procedure. Target amplification methods are among the oldest and best characterized nucleic acid amplification methodologies. Target amplification methods use enzymatic tools to increase the concentration of the target nucleic acids in the sample. Probe amplification methods increase the concentration of defined probe species when the target nucleic acid is present in the sample. At this writing, probe amplification methods have not been used to quantify viral nucleic acids. Signal amplification technologies (e.g., branched DNA and hybrid capture) do not generate new target or probe nucleic acids. Signal amplification methods use chemical and/or binding methods to amplify the signal (e.g., light or fluorescence) generated when specific nucleic acid hybridization occurs.

One of the greatest strengths and a major weakness of any nucleic acid amplification method is the exquisitely high sensitivity of these procedures. Nucleic acid amplification procedures can generate millions of DNA or RNA copies from template sequences. Sample splashes and contamination of specimens/reagents with nanoliter droplets of amplified products (amplicons) can produce a false-positive result. Therefore stringent amplicon control measures must be utilized to limit these problems.

False-negative results can occur when the specimen contains chemical or biological substances that inhibit the enzymatic amplification process. False-negative reactions can occur through a number of mechanisms. Specimens containing EDTA or other chelators can reduce the effective concentration of divalent cations that are necessary for the enzymatic amplification process. Proteases can degrade the amplification enzymes and the presence of RNase or DNase can degrade nucleic acid targets and/or primers. Finally, a wide variety of biological and chemical substances can directly inhibit the enzymes responsible for nucleic acid amplification. Nucleic acid amplification methods employing multiple enzymes generally have a broader inhibition profile than methods utilizing a single enzyme (NCCLS, 1995). Stringent sample preparation methods and specimen inhibition controls are necessary to minimize false-negative results.

PCR

Developed by researchers at the Cetus Corporation (Saiki et al., 1985; Mullis and Faloona, 1987), PCR is one of the oldest and best known methods for replicating specific DNA sequences in vitro. In its simplest form, the PCR procedure utilizes two 15- to 30-base oligonucleotide primers that are complementary to unique viral nucleic acid sequences located on opposite strands of the double-stranded nucleic acid. These primers are included in a reaction mixture containing the target nucleic acid, a heat-stable DNA polymerase, a defined solution of salts, and excess amounts of each of the four deoxynucleoside triphosphates. The mixture is then subjected to repeated cycles of defined temperature changes. These thermal changes facilitate the denaturation of the template DNA (94 to 97°C), the annealing of the primers to the target DNA (55 to 72°C), and the extension of the primers (72°C) so that the target DNA sequence is replicated (Fig. 1). During the next heating cycle, the strands separate and the original DNA strands and the newly synthesized DNA strands serve as templates for another round of DNA replication. Thus, the number of target DNA strands doubles with each thermal cycle. PCR procedures for infectious agents typically consist of 20 to 40 thermal cycles. These procedures produce a 10^5- to 10^6-fold increase in target nucleic acid concentrations within 3 to 4 h. The amplified DNA has traditionally been detected by capillary electrophoresis, solid-phase or solution hybridization, high-performance liquid chromatography, agarose gel electrophoresis with direct visualization of stained nucleic acids, or Southern blotting. Newer detection methods now use real-time amplification

TABLE 1 General summary of nucleic acid amplification testing methods

Method	Target(s)	Thermal cycling requirement	Sensitivity (organisms/ml)
PCR	DNA	Yes	5–50
NASBA	RNA	No	5–50
TMA	RNA	No	5–50
SDA	DNA	No	5–50
Real-time PCR	DNA	Yes	50–500
Cleavase invader	DNA	No	1,000–10,000
HPA	RNA	No	100,000
Hybrid capture	DNA RNA	No	3,000–7,000

and detection technologies and fluorescence resonance energy transfer (FRET) that significantly shorten the testing procedure.

PCR procedures can amplify only DNA targets. For RNA detection, the RNA must be converted to cDNA with a reverse transcriptase (RT) enzyme prior to amplification. Some of the newer thermostable DNA polymerases, such as *Tth*, have both RT and DNA polymerase activities and allow for cDNA generation and PCR amplification within a single tube (Myers and Gelfand, 1991). Such bifunctional polymerases greatly simplify RNA amplification procedures in the clinical laboratory.

The availability of universal master mixes and modern primer design software have greatly simplified in-house assay development. Primer design software like Primer Express (Applied Biosystems, Foster City, CA) use sophisticated selection algorithms that favor sequences that hybridize under universal thermal conditions. In addition to simplified assay development, the use of universal cycling conditions can minimize intra-assay variability in the clinical laboratory and allow the user to test for multiple analytes within a single batch.

Because PCR was the first target amplification technology to find widespread usage in the clinical laboratory, a wide variety of monoplex, multiplex (Edwards and Gibbs, 1994), and quantitative PCR procedures are available for clinical use. In addition, a portable, battery-operated, PCR detection system which utilizes FRET technology has been described (Belgrader et al., 1998; Belgrader et al., 1999) for field use.

PCR is an extremely sensitive and specific procedure. However, the PCR can be inhibited by a variety of substances including heme (Mercier et al., 1990; Ruano et al., 1992), heparin (Beutler et al., 1990; Holodniy et al., 1991), phenol (Katcher and Schwartz, 1994), polyamines (Ahokas and Erkkila, 1993), plant polysaccharides (Demeke and Adams, 1992), urine (Khan et al., 1991; Chernesky et al., 1997; Berg et al., 1997; Mahony et al., 1998; Toye et al., 1998), vitreous fluids (Wiedbrauk et al., 1995), and calcium alginate (Wadowski et al., 1994). The inhibition profile of the PCR procedure depends largely upon the type of polymerase

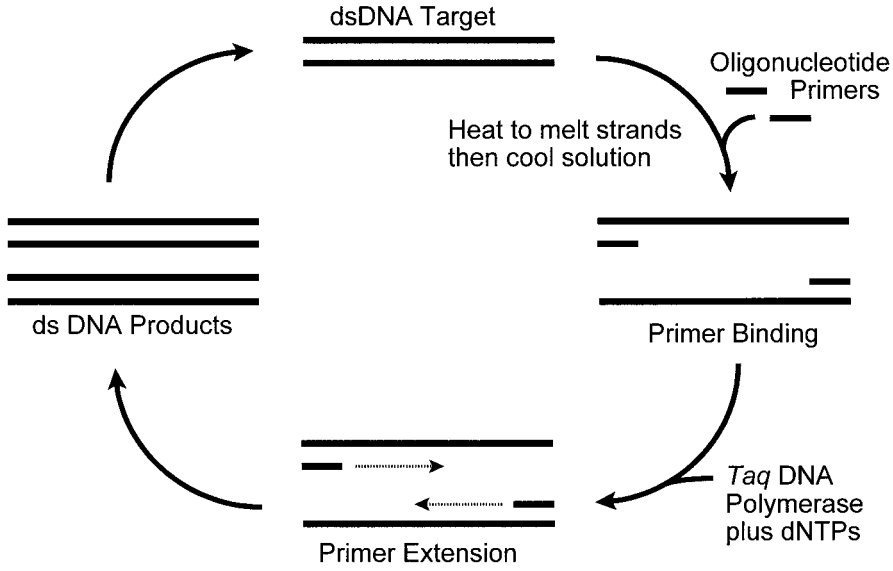

FIGURE 1 PCR. The dsDNA target (top) is heated to separate the strands. As the solution cools, the two oligonucleotide primers bind to opposite strands on the target DNA. The thermostable *Taq* DNA polymerase extends the primers according to the nucleotide sequence of the target DNA strand to produce dsDNA products. The old and new strands serve as templates for further DNA synthesis during the next cycle of heating and cooling. dNTPs, deoxynucleoside triphosphates.

used in the reaction (Wiedbrauk et al., 1995) and the purity of the nucleic acid to be amplified.

NASBA AND TMA

Nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA) are functionally identical isothermal target amplification procedures that are based upon the replication events that occur during retroviral transcription (Guatelli, 1990). In these procedures, RT, RNase H, and T7 RNA polymerase are used to generate new RNA targets via double-stranded DNA (dsDNA) intermediates (Fig. 2). Like PCR, NASBA and TMA utilize oligonucleotide primers that are complementary with the target nucleic acid sequences. However, at least one of these primers also contains a promoter sequence for T7 RNA polymerase. When the primer anneals to the target, the promoter end of the primer does not anneal with the target because it does not contain complementary sequences (Fig. 2). The annealed end of the primer is extended by RT. RNase H degrades the RNA in the RNA-DNA hybrid, allowing the second primer to bind to the cDNA. RT then extends the 5′ end of the primer. The resulting dsDNA contains complete, transcriptionally competent, T7 RNA polymerase promoter. T7 RNA polymerase binds to this promoter and produces 50 to 1,000 antisense RNA copies of the original target. These antisense transcripts are, in turn, converted to T7 promoter-containing double-stranded cDNA copies and used as transcription templates. This process continues in a self-sustained, cyclic fashion under isothermal conditions until components in the reaction mixture become limited or inactivated. In this procedure, each DNA template generates not one but many RNA copies, and transcription takes place continuously, without thermocycling.

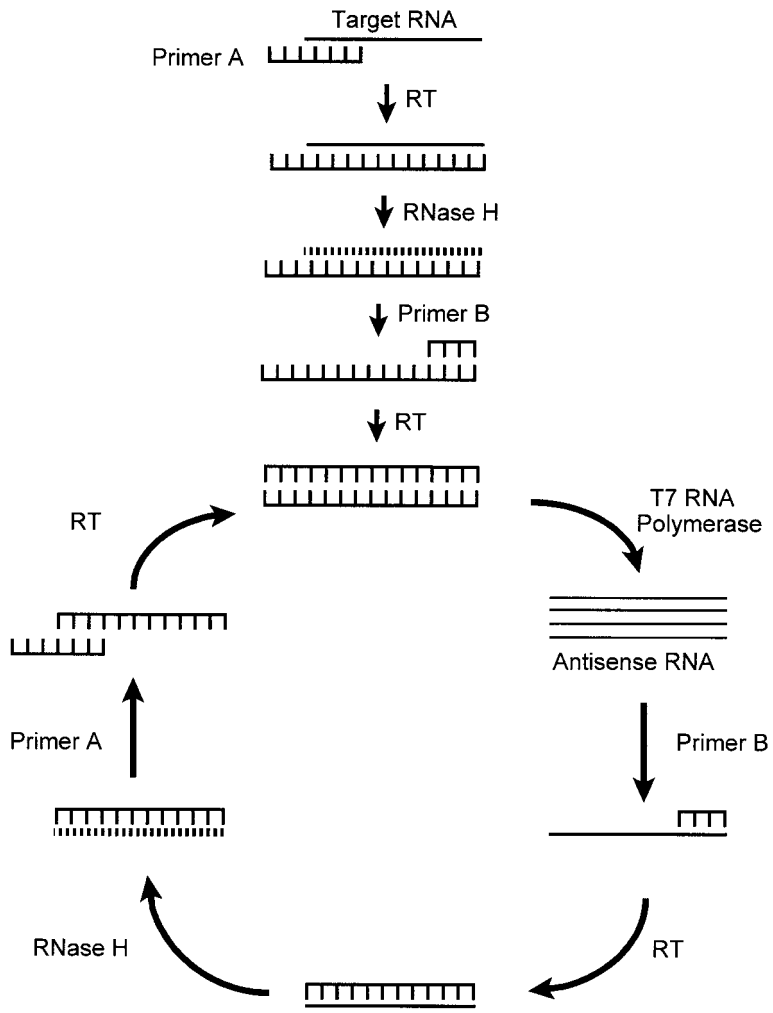

FIGURE 2 NASBA and TMA. In these procedures, primer A, containing the promoter sequence for the T7 RNA polymerase and a sequence complementary to the target RNA, binds to the target RNA strand (top). RT extends the primer according to the genetic sequence of the target strand, and RNase H degrades the RNA portion of the DNA-RNA hybrid molecule. Primer B binds to the complementary DNA, and RT extends the primer to make a complete, transcription-competent dsDNA intermediate. The T7 RNA polymerase generates 50 to 1,000 antisense (from the original RNA) RNA transcripts, each of which can be converted to transcription-competent dsDNA as before.

The resulting products can be detected by a variety of methods. NASBA and TMA can produce a 10^7-fold increase in the nucleic acid target in 60 to 90 min.

While NASBA and TMA can theoretically utilize both DNA and RNA targets, RNA targets are preferred for both methods. NASBA and TMA differ only in the number of enzymes used to catalyze the reaction. NASBA utilizes three separate enzymes, while TMA uses two. The TMA procedure employs a native RT that also has RNase H activity. Both procedures produce qualitative and quantitative results, and multiplex testing procedures have been developed. Instrumentation is now available for both methods, and a real-time detection system has recently been described that significantly shortens the assay time (Leone et al., 1998).

NASBA and TMA reactions can be inhibited by a variety of substances (Witt and Kemper, 1999), including urine (Mahony et al., 1998) and a variety of proteins and polysaccharides (personal observations). For this reason, most of the newer NASBA and TMA procedures utilize extensive nucleic acid purification methods prior to amplification. TMA procedures currently use target capture methods on paramagnetic beads, while the NASBA methods utilize the guanidinium extraction procedures described by Boom et al. (1990, 1999) to prevent inhibition.

SDA

The strand displacement amplification (SDA) method is an isothermal DNA amplification procedure developed by Walker et al. (1992). In its current configuration (Fig. 3), a primer containing a *Bso*B1 restriction site (5′-GGGCTC) hybridizes with complementary sequences on the target nucleic acid. The primer is extended by an exonuclease deficient (exo⁻) *Bst* DNA polymerase in the presence of dGTP, dATP, dTTP, and a derivatized dCTP containing an alpha-thiol group (dCTPαS) (Spargo et al., 1996). The resulting DNA synthesis generates a double-stranded *Bso*B1 recognition site, one strand of which contains phosphorothiolate linkages located 5′ to each deoxycytosine residue. The presence of the phosphorothiolate bond causes the *Bso*B1 restriction enzyme to nick the recognition site without cutting the complementary thiolated strand. The exo⁻ *Bst* polymerase fragment initiates another round of DNA synthesis at the nick. However, the DNA located downstream of the nick is not degraded because the exo⁻ Klenow fragment lacks 5′ exonucleolytic activity. Instead, the downstream DNA fragment is displaced as the new DNA molecule is synthesized. The displacement step regenerates the *Bso*B1 site. Nicking and strand displacement steps cycle continuously until the reaction mixture components become limited.

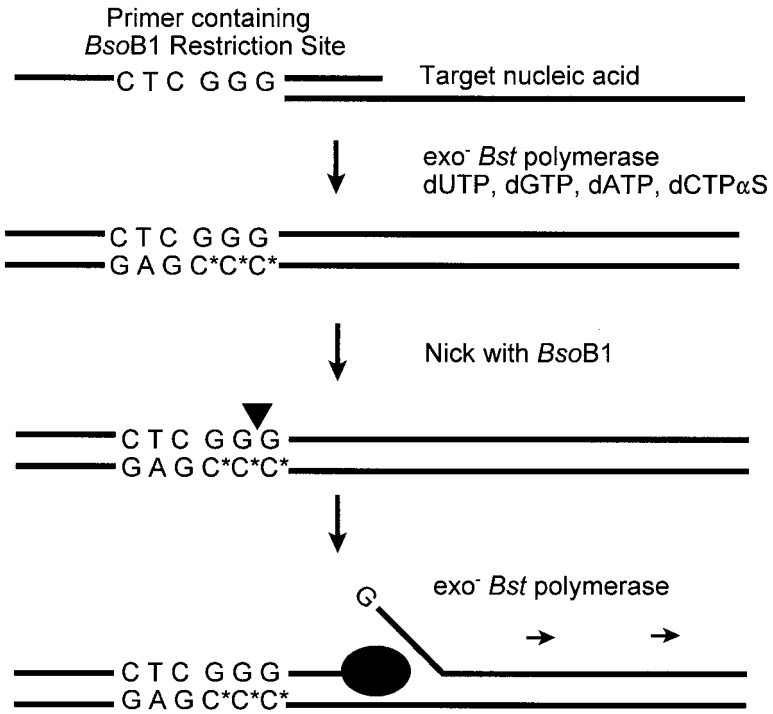

FIGURE 3 SDA. An oligonucleotide primer containing a *Bso*B1 restriction site (5′-CTCGGG) binds to the complementary target nucleic acid. The primer and target are extended by a thermostable, exonuclease-deficient (exo⁻) *Bst* DNA polymerase in the presence of dGTP, dATP, dUTP, and a dCTP that contains an alpha-thiol group (dCTPαS). The resulting DNA synthesis generates a double-stranded *Bso*B1 recognition site, with one strand containing 5′ phosphorothiolate linkages (shown as asterisks). *Bso*B1 nicks the strand without cutting the complementary thiolated strand, and the exo⁻ *Bst* polymerase extends the nucleic acid strand from the nick. The orignal nucleic acid is displaced rather than degraded because the DNA polymerase does not have 5′ exonucleolytic activity. The restriction site is regenerated by the polymerase. This linear amplification scheme becomes exponential when an antisense primer containing a *Bso*B1 site is added to the reaction mixture.

This linear amplification process can produce a new DNA target every 3 min (Walker et al., 1995). This amplification scheme becomes exponential when sense and antisense primers containing *Bso*B1 sites are used. The resulting procedure doubles the number of target sequences every 3 min until the reaction mixture components become rate-limiting. With the exception of an initial boiling step to denature the nucleic acids, SDA reactions are isothermal and are carried out at 50 to 60°C for 2 h. Performing SDA at stringent operating temperatures decreases the background amplification due to mispriming (Walker and Linn, 1996). Mispriming and background levels can be further reduced by using the single-stranded-DNA binding protein from gene 32 of bacteriophage T4 (Walker et al., 1996). This protein also enhances the ability to amplify longer (200- to 1,000-bp) target sequences (Walker et al., 1996). Real-time detection of SDA products using fluorescence polarization has been described; this allows for the detection of the cryptic plasmid of *Chlamydia trachomatis* in just 30 min (Little et al., 1999). Little is known about the inhibition profile of this procedure.

CLEAVASE INVADER ASSAY

The Cleavase Invader assay in its simplest form is a probe amplification system that utilizes two probes and a flap endonuclease (Cleavase) derived from an archebacterial species. In this assay, the invader probe is fully complementary with, and binds tightly to, the target nucleic acid (Fig. 4). The signal probe, which has two domains, is provided in vast excess. The 3′ end of the signal probe is complementary to the target sequence and binds immediately downstream from the invader probe. The 5′ end of the signal probe does not hybridize with the target. Once the invader and signal probes bind to the target, the flap endonuclease cleaves the noncomplementary portion of the signal probe. Because the hybridization reaction is performed at or near the melting temperature of the signal probe, the cleaved signal probe is replaced by an uncleaved signal probe 50 to 500 times/min. This procedure produces a 3,000- to 30,000-fold increase in signal in 60 min.

Early versions of the Invader assay used electrophoresis or plate-based hybridizations to detect the accumulation of the signal probe after it is cleaved by the flap exonuclease. Current Invader procedures utilize FRET cassettes to detect the accumulation of cleaved target probes in a homogeneous amplification and detection reaction (Ryan et al., 1999). In these FRET detection reactions, the cleaved flap serves as an Invader oligonucleotide that produces an overlapping structure with the FRET oligonucleotide. The displaced flap is recognized and cleaved by the Cleavase enzyme (Fig. 4). The flap that is excised from the FRET cassette contains the fluorophore. Cleavage separates the fluorophore from the quencher molecule, and fluorescent molecules accumulate in the reaction vessel as the reaction progresses. Similar to the initial reaction, the released 5′ flap and the FRET cassette cycle, resulting in amplified fluorescence signal. The initial and secondary reactions run concurrently in the same well.

Invader assays and other nucleic acid detection systems are inhibited by the presence of nucleases and proteases. Invader assays are sensitive to altered salt concentrations, and some magnetic bead extraction systems will require an additional wash step to prevent inhibition.

SIGNAL AMPLIFICATION

In contrast with target and probe amplification systems, signal amplification methods are designed to increase the signal strength of a detection system without increasing the number of target molecules. Signal amplification methods have several advantages over target amplification procedures. Signal amplification methods are generally simpler to perform and are not as susceptible to carryover contamination, and prior to the advent of real-time amplification and detection systems, quantitative signal amplification methods usually had significantly broader dynamic (linear) ranges. The first-generation signal amplification methods were less sensitive than target amplification procedures. However, the most sensitive signal amplification assays (e.g., branched DNA [bDNA]) are at least as sensitive as target amplification systems.

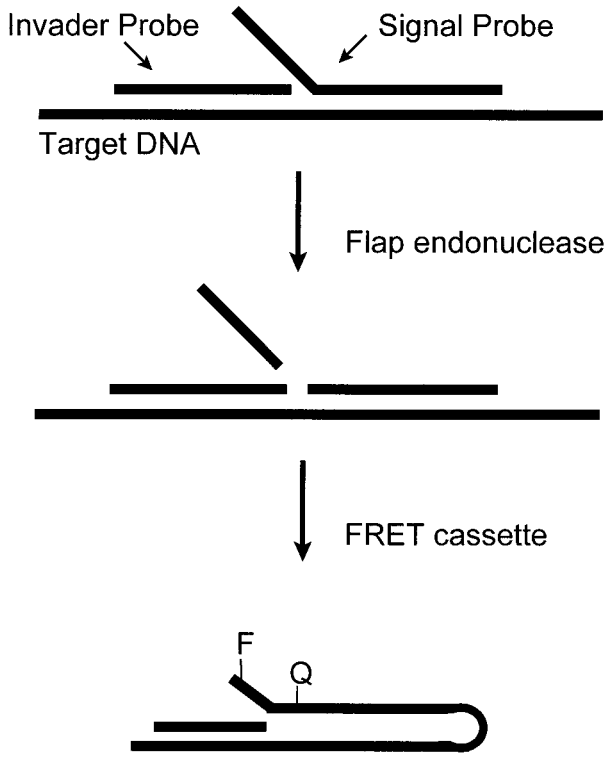

FIGURE 4 Cleavase Invader assay. The invader probe and the signal probe hybridize adjacent to each other so that a portion of the signal probe does not hybridize to the target. The flap endonuclease (Cleavase) excises the unhybridized portion of the signal probe. The signal probe dissociates because the reaction is performed at or near the melting temperature for this probe. Another signal probe hybridizes, and the cycle repeats. The cleaved portion of the signal probe serves as an invader probe in the FRET cassette, and hybridization with the cassette produces an overlapping structure. The Cleavase enzyme excises the overlapping structure and separates the fluorophore (F) from the quencher (Q). The unquenched fluorophores accumulate in the reaction vessel.

EIA-BASED DETECTION

Early attempts to automate nucleic acid assays and reduce the turnaround time and labor content of these procedures relied heavily upon enzyme immunoassay (EIA) technologies. EIA methods are often used to detect amplified DNA products because automation is readily available and familiar to those who work in the clinical laboratory. The most common EIA method for detecting amplified target DNA is shown in Fig. 5. In this procedure, biotinylated capture

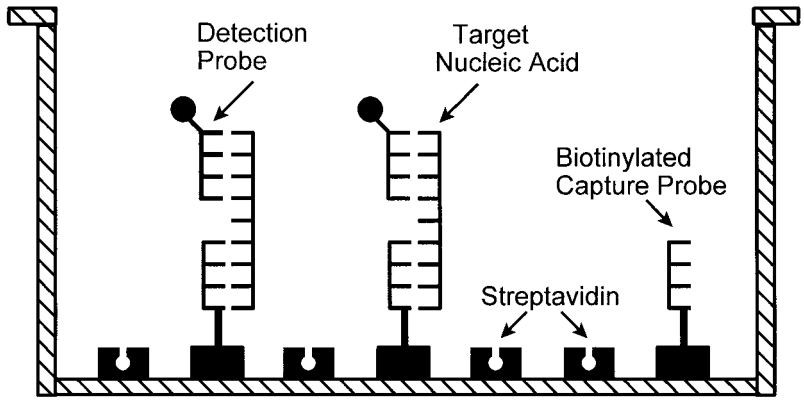

FIGURE 5 Typical EIA detection system. The target nucleic acids are heated and allowed to hybridize with biotinylated capture probes. The resulting mixture is placed into a microtiter plate well containing immobilized streptavidin (dark rectangles). The unhybridized nucleic acids are washed away, and a labeled detection probe is allowed to hybridize. After another wash, an appropriate substrate is added. A signal (color, fluorescence, or light, etc.) is generated when the target nucleic acid is present in the sample.

probes and enzyme-labeled detection probes are allowed to hybridize (sequentially or simultaneously) to the target DNA in a streptavidin-coated microtiter plate well. PCR products can be captured directly by using biotinylated primers. After hybridization is complete, the unbound probes are removed by several high-stringency washes using a standard microtiter plate washer. Chromogen and substrate are added to the wells, and the absorbance of the solution is measured using a standard microtiter plate spectrophotometer. These procedures are qualitative, but the concentration of target DNA is usually proportional to the final absorbance of the solution.

EIA detection systems are sensitive, relatively fast, and easy to perform. EIA systems utilizing nucleic acid probes are very specific, and primer-dimers and other nonspecific amplification products are not detected. The multiple washing steps tend to minimize any inhibition caused by the specimen. A number of procedural modifications have been described for different solid supports (paper or latex, etc.) and detection systems (e.g., fluorescence or chemiluminescence).

When plate-based microtiter systems are used to detect amplified nucleic acids, the laboratory must take special precautions to minimize amplicon contamination of the laboratory. Plate-based systems can produce aerosols when the amplified nucleic acids are aspirated and when the plates are washed.

Hybrid Capture

An interesting modification of the EIA procedure has been introduced by Digene Diagnostics, Inc. (Silver Spring, MD), for use with their RNA probe systems. In this system, the target DNA is denatured and allowed to hybridize to an unlabeled RNA probe (Fig. 6). The hybridization mix is then transferred to an antibody-coated microtiter plate, and the DNA-RNA hybrids are captured. The unbound materials are removed by washing, and the DNA-RNA hybrids are detected using a unique alkaline phosphatase-labeled monoclonal antibody that is specific for DNA-RNA hybrids. After several washes, a dioxitane substrate is added to the well, and the chemiluminescent signal is measured with a luminometer. The use of a chemiluminescent substrate and the ability to bind multiple antibodies to each hybrid significantly enhances the signal generation properties of this assay. Using 10-fold dilutions of two Eurohep hepatitis B virus reference plasma specimens, Kessler et al. (1998) reported that the detection limit of the version 1 hybridization assay was 10^6 to 10^7 copies/ml compared with 10^3 copies/ml with the Amplicor PCR assay. Cullen et al. (1997) reported that the analytical sensitivity of the Digene hybrid capture 2 test

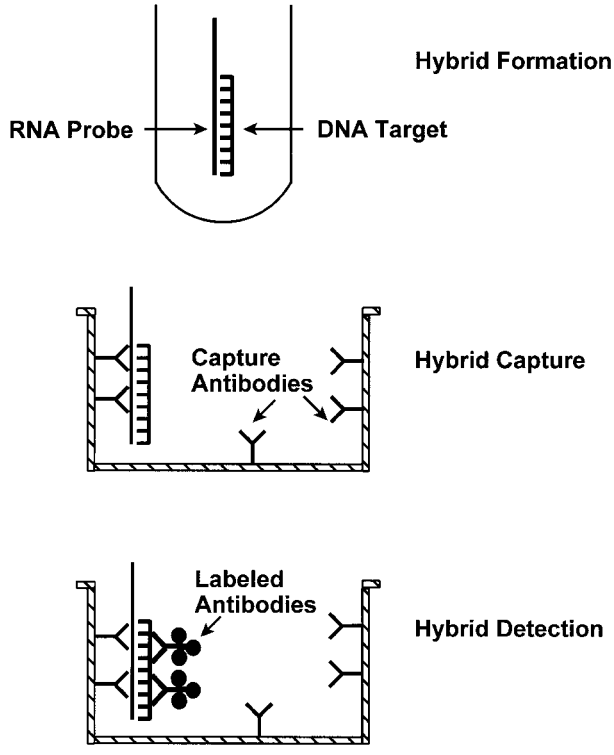

FIGURE 6 Hybrid capture. The target DNA is denatured and allowed to hybridize to a large unlabeled RNA probe. The DNA-RNA hybrids are captured in a microtiter plate well by immobilized, hybrid-specific antibodies. Unbound materials are removed with a wash step. Labeled monoclonal antibodies to the DNA-RNA hybrid are added, and they bind to the entire length of the hybrid. After another wash, the chemiluminescent substrate is added. Light is generated if the target DNA was present in the sample.

for herpes simplex virus was 5×10^3 to 1×10^4 copies per assay. The minimum amount of human papillomavirus (HPV) DNA that gives a positive result in the hybrid capture 2 assay is 1.0 pg of HPV DNA/ml. This yields an effective limit of detection of about 4,700 HPV copies/ml (Sandri et al., 2006). Little is known about the inhibition profile for the hybrid capture procedure, but care must be taken during the extraction and hybridization procedures to prevent the introduction of RNases that could degrade the RNA target probe.

bDNA ASSAY

The bDNA system developed by Chiron Corporation (Emeryville, CA) is one of the most powerful signal amplification systems described to date (Urdea et al., 1987). This procedure (see chapter 15, this volume) utilizes an intricate network of oligonucleotide capture probes, target probes, novel branched secondary probes, and short, enzyme-labeled, tertiary probes to capture the target and produce a signal. bDNA methods can be used to detect viral nucleic acids at their naturally occurring concentrations without nucleic acid replication. This method has an extremely broad dynamic range that appears to be well suited to nucleic acid quantitation. The newest bDNA methods are almost as sensitive as PCR, detecting as few as 50 human immunodeficiency virus copies/ml of plasma (Collins et al., 1997).

HPA

The hybridization protection assay (HPA) (Gen-Probe, San Diego, CA) was one of the first probe assays to receive FDA clearance for clinical diagnostic use. HPA utilizes a novel chemiluminescent technology to amplify the signal while minimizing background noise (Fig. 7). HPA employs a chemiluminescent acridinium ester label that is covalently coupled to the oligonucleotide probes via an acid-sensitive ether bond (Arnold et al., 1989). Once the esters are hydrolyzed, the label becomes permanently nonluminescent. Probes that are bound to target nucleic acids are protected from hydrolysis and retain their chemiluminescence. While this is a qualitative assay, the amount of chemiluminescence produced in the HPA is proportional to the amount of probe-target hybrid formed. HPA, by itself, has significantly less sensitivity than PCR tests. The commercial HPA procedure for *Chlamydia trachomatis*, for instance, is very specific but has no more sensitivity than commercially available EIAs (Schachter, 1997).

REAL-TIME DETECTION OF NUCLEIC ACIDS

SYBR Green I

SYBR green was the first of a new generation of DNA stains introduced by Molecular Probes, Inc. (Eugene, OR), in 1995 as a safer (Singer et al., 1999) and more sensitive alternative to ethidium bromide for staining agarose gels. SYBR green I is a fluorescent dye that binds to the minor groove of dsDNA. Unbound SYBR green barely fluoresces in solution, but DNA binding causes conformational changes in the dye that increases the fluorescence intensity by 100-fold.

Applied Biosystems, Inc. (Foster City, CA), then developed conditions that permitted the use of SYBR green I dye in PCRs without PCR inhibition. This modification provided a simple and inexpensive approach to real-time amplification and detection of PCR product wherein the SYBR green signal increased as the amount of dsDNA increased. SYBR green detection proved to be very sensitive, and it proved to be simple and economical to apply to well-established PCR assays because it did not require additional fluorescence-labeled oligonucleotides. The disadvantage to this procedure is that SYBR green will bind to any dsDNA in the reaction, including primer-dimers and other nonspecific reaction products.

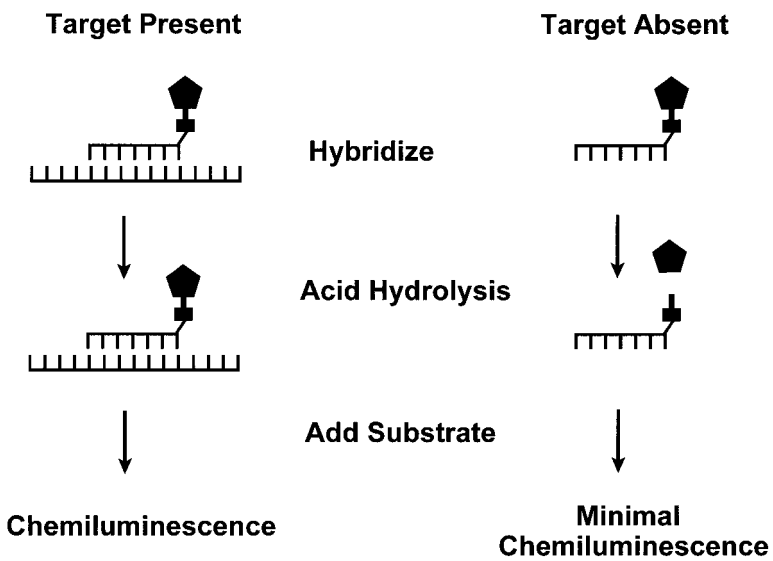

FIGURE 7 HPA. The oligonucleotide detector probe contains a chemiluminescent acridinium ester that is covalently attached to the probe via an acid-sensitive ether bond (top right). Complementary base pairing of the probe to the target protects the ether bond from acid hydrolysis. In the absence of base pairing, the ether bond is hydrolyzed and the label becomes permanently nonluminescent.

Therefore, SYBR green PCR procedures must be must be carefully optimized to prevent mispriming and primer dimers. In addition, the presence of a specific amplified product is usually confirmed by melt curve analysis.

FRET System

FRET is a distance-dependent interaction between two dye molecules wherein excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon (Cardullo et al., 1988). Regular (non-FRET) fluorescence occurs when a fluorescent molecule (fluorophore) absorbs electromagnetic energy of one wavelength (the excitation frequency) and reemits that energy at a different wavelength (the emission frequency). For the combined FRET effect, the emission peak of the donor must overlap with the excitation peak of the acceptor. In FRET, light energy is added at the excitation frequency for the donor fluorophore, which transfers some of this energy to the acceptor. The acceptor, in turn, reemits the light at its own (lower frequency) emission wavelength. Depending upon the type of detectors used, FRET acceptors can function as fluorescence quenchers (detector cannot detect the lower wavelength acceptor emissions) or as a primary detection system when the donor and acceptor are adjacent (detector is designed to detect the lower wavelength acceptor emissions but not the higher wavelength donor emissions).

Real-time FRET systems for detecting amplification or hybridization events are used by an increasing number of molecular diagnostics laboratories. The advantage of these methods is their rapid throughput and reduced labor costs versus more traditional PCR and hybridization methods. These homogeneous amplification and detection procedures also minimize amplicon contamination of the laboratory because reaction vessels containing amplified nucleic acids are not opened.

A wide variety of instrumentation is available which can measure the fluorescence intensity after each thermal cycle. Most of these instruments can detect and quantify several colored fluorescent dyes simultaneously. The use of multiple fluorophores allows for the simultaneous detection of multiple agents and/or internal controls within the same reaction vessel. FRET technology has been used in a variety of nucleic acid detection methodologies, including PCR (Livak et al., 1995), SDA (Walker et al., 1996), TMA/NASBA (Loens et al., 2006), and Cleavase Invader assays (Ryan et al., 1999). The rapid throughput of this system, the ability to quantify nucleic acids in a single tube, and the applicability to several amplification systems make the FRET system a valuable tool for the diagnosis of viral infections despite its slightly lower sensitivity levels.

Hydrolysis (TaqMan) Probes

One real-time FRET system is the TaqMan detection system from Roche Molecular Diagnostics (Indianapolis, IN) and Perkin-Elmer (Waltham, MA). This method, which is used in conjunction with PCR, employs two standard PCR primers and an internal reporter oligonucleotide. The oligonucleotide has a fluorescent reporter molecule coupled to the 5′ end (Fig. 8) and a quencher molecule at the 3′ end (Livak et al., 1995). When the probe is intact, the proximity of the quencher molecule suppresses the fluorescence of the reporter molecule (Holland et al., 1991). During PCR, the reporter probe is digested by the 5′→3′ nuclease activity of the polymerase. This digestion separates the reporter and quencher molecules, causing a significant increase in the background fluorescence in the tube (Lee et al., 1993; Livak et al., 1995). Cleavage also removes the probe from the target strand, allowing primer extension to continue normally. Additional reporter dye molecules are cleaved from their respective probes with each thermal cycle, causing a cumulative increase in fluorescence intensity that is proportional to the amount of amplicon produced. The ability to monitor the real-time progress of the PCR allows the laboratory to quantify nucleic acids based upon the time it takes for the fluorescence to reach an arbitrary threshold. The higher the starting copy number of the nucleic acid target, the sooner the arbitrary threshold is reached.

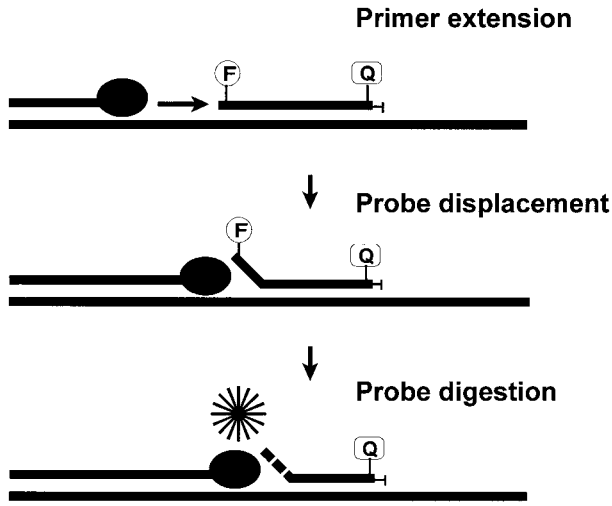

FIGURE 8 Hydrolysis (TaqMan) probes. This method utilizes a reporter oligonucleotide that has a fluorescent dye (F) covalently coupled to the 5′ end and a quencher dye (Q) on the 3′ end. For PCR procedures, the reporter probe hybridizes internally to the flanking PCR primers. As the upstream primer is extended, the reporter oligonucleotide is displaced, then digested by the 5′→3′ nuclease activity of the polymerase (black oval). Digestion separates the reporter and quencher molecules and allows the reporter molecule to fluoresce strongly. Fluorescent reporter molecules accumulate with each amplification cycle.

The FRET procedure is slightly less sensitive than standard PCR (Ryncarz et al., 1999), principally due to the extremely small sample size. Kawai et al. (1999) and Martell et al. (1999) reported that the TaqMan system had a lower limit of detection of 1 to 2 × 10^3 copies/ml for hepatitis C virus. However, both reports state that the TaqMan method has a dynamic (linear) range of 5 logs within a single reaction tube, which is significantly better than traditional PCR methods.

An advance in the TaqMan technology has been the addition of a DNA minor groove-binding moiety at the 3′ end of the TaqMan probes (Kutyavin et al., 2000), which increases their stability and specificity. This minor groove-binding modification allows the probes to be shorter—about 13 to 20 bp long—and less expensive to make. Shorter probes are also more sensitive to single base mismatches (Kutyavin et al., 2000).

Hybridization Probes

The hybridization probe format utilizes two specifically designed, sequence-specific oligonucleotide probes that are labeled with different dyes. The sequences of the probes are

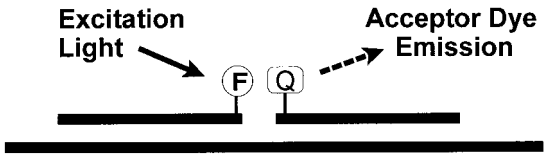

FIGURE 9 Hybridization probes. The hybridization probe format uses two oligonucleotides. The donor oligonucleotide is labeled at the 3′ end with a fluorescent dye (F), and the acceptor oligonucleotide (Q) is labeled at the 5′ end with a dye whose excitation frequency overlaps with the emission frequency of the donor dye. The probes are designed so that they hybridize in a head-to-tail arrangement on the target nucleic acid. If the target is present, the donor and acceptor dyes are in close proximity. FRET occurs when donor dye is excited by the light source. Light emitted by the donor dye then excites the acceptor dye, and the longer wavelength light emitted by the acceptor dye is detected by the instrument.

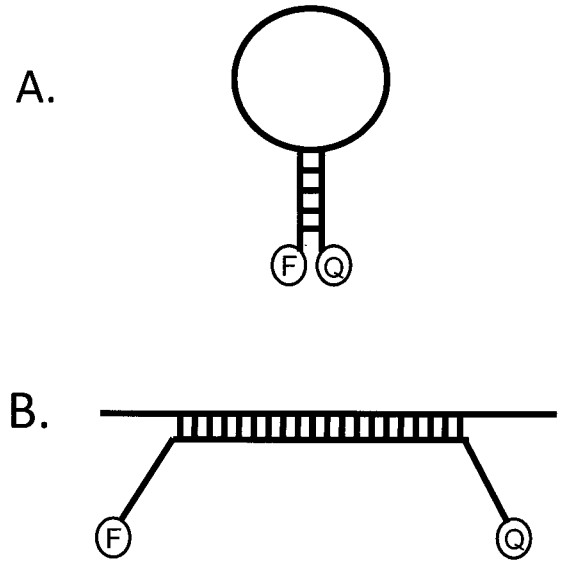

FIGURE 10 Molecular beacon. Molecular beacons are stem-loop structures where the ends of the oligonucleotide are self-complementary and the center portion of the molecule is complementary to the target sequence (A). Self-annealing of the ends brings the fluorophore (F) and quencher (Q) dyes into close proximity, and the molecule will not fluoresce. In the presence of the target DNA (B), hybridization of the loop sequence is favored and the fluorophore and the quencher molecules are separated. The molecular beacon will fluoresce strongly in the presence of the target DNA.

selected so that they can hybridize to the target sequences on the amplified DNA fragment in a head-to-tail orientation, thus bringing the two dyes into close proximity (Fig. 9). The donor dye (e.g., fluorescein) is excited by the blue light source and emits a longer-wavelength green fluorescent light. When the donor and acceptor dyes are in close proximity, the green fluorescent light energy from the donor dye excites the acceptor dye attached to the second hybridization probe. The acceptor dye then emits fluorescent light at its (still longer) emission wavelength. Fluorescence detectors used for hybridization probe assays are designed to detect light from the acceptor molecule and not the excitation light or the emission fluorescence from the primary probe. The energy transfer from the donor to the acceptor depends greatly on the spacing between the two dye molecules. Energy is only transferred efficiently if the molecules are in close proximity (between 1 to 5 nucleotides). Like the other real-time detection systems, the amount of fluorescence emitted is directly proportional to the amount of target DNA generated during the PCR.

Molecular Beacons and Scorpion Probes

Molecular beacons were first introduced by Tyagi and Kramer in 1996 (Tyagi and Kramer, 1996). Molecular beacons are oligonucleotides that, like hydrolysis probes, have a fluorophore on one end and a quencher molecule at the other (Fig. 10). Molecular beacons contain two functional moieties—a region that is complementary to the proposed target sequence and flanking sequences that contain a self-complementary region of five to six nucleotides. In the absence of the target, the complementary parts of the probe hybridize together, forming a stem-loop structure (Fig. 10). This structure brings the fluorophore and the quencher into close proximity, and the probe produces very little signal. In the presence of the target, the probe region of the molecular beacon hybridizes and the binding separates the fluorophore from the quencher. The amount of fluorescence in the reaction mixture increases as the concentration of the target increases, thus allowing molecular beacons to be used for qualitative and quantitative real-time assays. Molecular beacon assays that detect multiple targets through the accumulation of different colored fluorescent dyes have been described previously (Piatek et al., 1998; Tan et al., 2004; Marras et al., 2006). Scorpion probes were first described by David Whitcombe and coworkers in 1999 (Whitcombe et al., 1999). Scorpion probes are similar to molecular beacons in that they are stem-loop structures with a fluorophore on one end of the self-complementary step and a quencher on the end of the other stem. When the target is absent, the self-complementary stem structure brings the fluorophore in close approximation to the quencher, so no signal is generated. Unlike molecular beacons, Scorpions function both as a PCR primer and a probe. Scorpions have a primer sequence at the 5′ end. This "tail" hybridizes to the extension product of the primer, thereby separating the fluorophores from the quencher molecule.

SOLID AND LIQUID ARRAY-BASED SYSTEMS

A DNA microarray is an orderly arrangement of microscopic DNA spots (usually oligonucleotides) on a solid support, providing a medium for hybridizing known and unknown nucleic acid samples. Most microarrays use fluorescent probe systems to signal a specific hybridization event. Microarray readers contain lasers to excite the fluorophores, a microscope to examine the microscopic spots, and a digital camera to record the fluorescence levels. Computer programs are used to analyze the digital images and determine positive or negative status or the level of gene expression. Solid-phase arrays are principally used for gene expression profiling and hybridization sequencing, but they are increasingly being used for simultaneous detection of multiple viral analytes (Khodakov et al., 2008), viral genotyping (Brown et al., 2007; Crum et al., 2004; Moore et al., 2007; Tempfer et al., 2007), and identifying new viruses (Kistler et al., 2007).

Two microbead-based liquid arrays have been described recently for the detection of multiple human respiratory viruses in clinical specimens (Mahony et al., 2007; Lee et al., 2007; Brunstein and Thomas, 2006). Briefly, these procedures consist of amplification of viral cDNAs by PCR, labeling of the PCR products with virus-specific tags and site-specific biotins by target-specific extension (TSE) of tagged primers, capture of the tagged TSE products by color-addressed microspheres through the hybridization of each tag to a complementary oligonucleotide that is attached to surface of the microsphere, binding of fluorescent streptavidin-phycoerythrin to the biotin on the TSE products, and reading of fluorescent signals on each microsphere using the Luminex xMap 100 instrument (Luminex Corporation, Austin, TX). These fluid-based microarrays are being used in an increasing number of laboratories to expand the range of viruses detected and to unify several viral detection platforms.

CHOOSING AN AMPLIFICATION SYSTEM

Choosing an appropriate amplification system can be confusing because few tests have been "approved" by the FDA, and an increasing number of vendors are entering the market. FDA approval is a critical parameter for some laboratories because they do not have sufficient staff and/or expertise to develop new assays and perform quality assurance monitoring of reagents and intermediate solutions. Other laboratories prefer FDA-approved products because they can be inspected using the Microbiology Checklist from the College of American Pathologists. Laboratories using in-house-developed methods must use the more extensive Molecular Pathology Checklist from the College of American Pathologists.

Test system choices can be difficult even when choosing among FDA-approved procedures. A number of factors influence test system choices, including the expected test menu, anticipated test volumes, technical expertise, space constraints, and whether the laboratory has previously purchased nucleic amplification and detection equipment. The patient population is an important factor because some commercial assays may work well in one patient population and have significant limitations in another.

Anticipated test volumes also have an important impact upon test selection. Small-volume laboratories (<30 specimens/day) may elect to use LightCycler (Roche Applied Science, Indianapolis, IN), SmartCycler (Cepheid Corporation, Sunnyvale, CA), or GeneXpert (Cepheid Corporation) systems that are designed for smaller batch sizes. Higher-volume laboratories may need more than one thermocycler and/or detection system to test all their specimens on one shift.

Laboratories that develop in-house assays are currently limited to PCR and NASBA methodologies. Other amplification methods described in this chapter are part of closed assay systems whose manufacturers discourage or prohibit in-house development. The most popular in-house method is PCR. PCR and its derivatives (e.g., RT-PCR, in situ PCR, and FRET detection, etc.) have been used to detect and quantify nucleic acid targets by an increasing number of clinical laboratories. Several manufacturers are assisting in the development of in-house PCR tests by making analyte-specific reagents that can be used as components for in-house developed procedures. Use of analyte-specific reagents can significantly shorten development time. Laboratories performing certain PCR procedures for clinical purposes must obtain a license from Roche Diagnostics and pay a royalty on all clinical tests. Laboratories using the TaqMan procedure must pay TaqMan real-time PCR royalties for clinical assays.

Nucleic acid extraction is a critical component in any nucleic acid assay, and a significant proportion of the total testing time is dedicated to extraction procedures. Nucleic acid extraction must be able to remove inhibitory and interfering substances without significantly altering the amount or quality of the target nucleic acids. In general, cruder extraction procedures can be paired with robust detection systems, but they may not work for all procedures. Nucleic acid amplification methods that employ multiple enzymes are generally more sensitive to the presence of inhibitory substances than methods utilizing one enzyme (NCCLS, 1995). Automated extraction systems can improve extraction efficiency and reproducibility, but they are expensive and require dedicated bench space. For small-volume laboratories, systems like the GeneXpert (Cepheid Corporation) may be ideal because these systems perform extraction, amplification, and detection with few manipulations and in a small footprint. However, GeneXpert tests are more expensive than other methods and the test menu is limited.

An increasing number of laboratories are starting to perform nucleic acid tests, and detection is still in its infancy. Choosing among test methodologies will be difficult until more tests are available commercially. Until then, molecular virology laboratories must utilize multiple methods to detect viral agents in clinical specimens.

CONCLUSIONS

Virus detection is increasingly important for the diagnosis of viral disease, clinical management of patients on antiviral therapies, detecting the emergence of drug resistance, and reducing length of hospitalization. To be useful, however, virus detection information must be delivered within a clinically relevant time frame. Many of the nucleic acid detection methods mentioned in this chapter have significantly reduced the time-to-result for virus detection, thereby contributing to improved patient management. Nucleic acid detection methods also have made significant improvements in our ability to detect fastidious and slow-growing viruses (e.g., human parvovirus, Epstein-Barr virus, and certain enteroviruses), viruses that are dangerous to amplify in culture (e.g., human immunodeficiency virus and certain hemorrhagic fever viruses), and viruses that are present in low concentrations (Wiedbrauk and Hodinka, 1998). More importantly, nucleic acid methods have expanded the role of the clinical virology laboratory by allowing laboratories to detect viruses that do not grow in culture (e.g., hepatitis B virus, hepatitis C virus, HPV). While nucleic acid detection methods will never completely replace culture and direct fluorescent antibody methods, nucleic acid detection methods will continue to play an important role in the detection and monitoring of viral diseases.

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Quantitative Molecular Techniques

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The development of quantitative nucleic acid amplification techniques created new opportunities for the clinical laboratory to impact the diagnosis and management of patients with viral diseases. These techniques provide important information that can be used to predict disease progression, distinguish symptomatic from asymptomatic infection, monitor the development of antiviral resistance, and assess the efficacy of antiviral therapy. Prior to their development, virologists were limited either to laborious culture methods available only in research laboratories or insensitive antigen detection assays to measure viral load. Viral load first appeared in the scientific literature in 1987, in a paper by Jonas Salk (Salk, 1987), proposing that viral load in human immunodeficiency virus type 1 (HIV-1)-infected individuals could be reduced by boosting the immune response, leading to reduced morbidity, mortality, and disease transmission. Viral load assays assess the overall virus replicative activity that reflects the underlying disease process usually by quantification of the viral nucleic acid in the blood (Dailey and Hayden, 1999).

Viral load testing in HIV-1 infection is the best example of how quantitative molecular techniques have increased our understanding of a disease process and improved patient care. HIV-1 viral load is a powerfully predictive test for disease progression and is currently used to start, monitor, and change antiretroviral therapy (Hammer et al., 2006). Viral load assays have a similar impact on the care of patients with other chronic viral infections for which the therapy is currently not optimal, such as hepatitis B and hepatitis C. Viral load assays also provide important information in distinguishing active from latent herpesvirus infections in immunocompromised patients and in diagnosing and monitoring BK polyomavirus-associated nephropathy in renal transplant recipients.

In this chapter, the quantitative molecular techniques that serve as the basis of viral load assays will be described along with key issues and important variables that affect assay performance. The important clinical applications of these assays will be reviewed. The reader is directed to the Clinical Laboratory and Standards Institute's guideline *Quantitative Molecular Methods for Infectious Diseases* (National Committee for Clinical Laboratory Standards, 2003) and chapter 8 of *Molecular Microbiology*: *Diagnostic Principles and Practice*, 2nd ed. (Shepley and Wolk, 2004) for more information on implementing these assays in the clinical laboratory.

HISTORY

The first description of the PCR by Mullis and colleagues was a milestone in biotechnology and heralded the beginning of molecular diagnostics (Saiki et al., 1988). The first applications of PCR in clinical virology were qualitative in nature and provided very sensitive methods for detection of viral nucleic acids in clinical specimens. However, semiquantitative and quantitative applications of PCR for viral gene analysis followed, as it was realized that a linear relationship existed between the amounts of input template and amplification product.

Initial attempts at quantitative PCR involved simple quantitation of the amplification product (Oka et al., 1990; Warren et al., 1991). However, since PCR results in an exponential amplification of the initial target copy number, small differences in amplification efficiency from sample to sample lead to large and unpredictable differences in the amount of final reaction product. The unpredictable variability of individual amplification reactions prevents reliable quantitation of nucleic acids with this approach.

Other early attempts at quantitative PCR used limiting dilutions of samples (Simmonds et al., 1990; Brillanti et al., 1991). In limiting-dilution PCR, a series of dilutions of the sample are made, PCR is performed on multiple aliquots of each dilution, and the mean number of target molecules can be calculated based on the percentage of aliquots that are PCR positive at a given dilution. The precision of this approach is limited by the precision with which the dilution series is made. Furthermore, limiting-dilution PCR is impractical for routine use because of the large number of PCRs that must be performed to quantitate each sample.

The first attempts to control for sample-to-sample variation in amplification efficiency used coamplification of internal reference templates. Single-copy cellular genes (Kellog et al., 1990) or ubiquitously expressed transcripts (Noonan et al., 1990) have been used as internal reference templates. In concept, variables influencing the amplification efficiency should affect both templates similarly. In practice, however, different PCR templates may have very different thermodynamics and amplification efficiencies. The different template sequences may influence the amount of both products in an unpredictable manner. Also, this approach is limited in virological applications in that it cannot be used to quantitate extracellular templates, such as viruses, in serum or plasma samples.

Quantitative competitive PCR (cPCR) was the first truly quantitative PCR method developed and is used widely for quantitation of viral nucleic acids (Gilliland et al., 1990; Piatak et al., 1993). cPCR relies on the inclusion of an internal control competitor in each reaction mixture. The competitor molecules have the same primer binding sequences as the target molecule and are similar in size and base composition to the target but are distinguishable from it. Therefore, the efficiency of each reaction can then be normalized to the internal competitor. A known amount of competitor is added to each sample, and the ratio of PCR products reflects the ratio between the initial amounts of competitor and target.

Real-time PCR methods were first described in the late 1980s (Cardullo et al., 1988; Lee et al., 1993; Heid et al., 1996). In real-time PCR, target amplification and detection occur simultaneously in the same tube. Up until this time, PCR methods were heterogeneous, with amplification and product detection taking place separately. These methods employ novel fluorogenic probes or fluorescent dyes to monitor PCR product as it accumulates and require special thermal cyclers with precision optics that can monitor the fluorescence emission from the sample wells. The most commonly employed fluorogenic probes rely on fluorescence resonance energy transfer (FRET) between fluorophores or between one fluorophore and a dark-hole nonfluorescent quencher which disperses energy as heat rather than fluorescence (Didenko, 2001). In addition to being homogeneous, real-time methods are also kinetic, since PCR product is measured as it accumulates rather than at the end of the thermal cycling. Real-time methods offer the advantages of increased dynamic range, simplicity, reduced analysis time, and diminished risk of contamination.

Although PCR is the best developed and most widely used nucleic acid amplification strategy, other strategies have been developed and several serve as the basis of quantitative assays for viral nucleic acids. Quantitative assays based on nucleic acid sequence-based amplification (NASBA), branched DNA (bDNA), and hybrid capture are commercially available.

METHODS USED

PCR

PCR is a simple, in vitro chemical reaction that permits the synthesis of essentially limitless quantities of a targeted nucleic acid sequence. This is accomplished through the action of a DNA polymerase that, under the right conditions, can copy a strand of DNA. At its simplest, a PCR consists of target DNA, a molar excess of 2 oligonucleotide primers, a heat-stable DNA polymerase, an equimolar mixture of deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), $MgCl_2$, KCl, and a Tris-HCl buffer. The 2 primers flank the sequence to be amplified, typically <100 to several hundred bases, and are complementary to opposite strands of the target.

To initiate a PCR, the reaction mixture is heated to separate the two strands of target DNA and then cooled to permit the primers to anneal to the target DNA in a sequence-specific manner. The DNA polymerase then initiates extension of the primers at their 3′ ends toward one another. The primer extension products are dissociated from the target DNA by heating. Each extension product, as well as the original target, can serve as a template for subsequent rounds of primer annealing and extension.

A PCR cycle consists of three steps, denaturation, annealing, and extension. At the end of each cycle, the PCR products are theoretically doubled. Thus, after n PCR cycles, the target sequence can be amplified 2^n-fold. The whole procedure is carried out in a programmable thermal cycler that precisely controls the temperature at which the steps occur, length of time that the reaction mixture is held at the different temperatures, and the number of cycles. Ideally, after 20 cycles of PCR, a millionfold amplification is achieved, and after 30 cycles, a billionfold amplification has occurred. In practice, the amplification may not be completely efficient due to failure to optimize the reaction conditions or the presence of inhibitors of the DNA polymerase. In such cases, the total amplification is best described by the expression $(1 + e)^n$ where e is the amplification efficiency ($0 \leq e \leq 1$) and n is the total number of cycles.

A variety of PCR-based strategies have been developed to accurately quantitate DNA and RNA targets in clinical specimens, including simple quantitation of the amplification product (Oka et al., 1990; Warren et al., 1991), limiting dilutions of samples (Simmonds et al., 1990; Brillanti et al., 1991), coamplification of internal reference templates (Kellog et al., 1990; Noonan et al., 1990), and cPCR (Gilliland et al., 1990; Piatak et al., 1993). At the time, it was generally accepted that cPCR is the most reliable and robust approach to gene quantitation (Clementi et al., 1993).

The basic concept behind cPCR is the coamplification in the same reaction tube of two different templates of equal or similar lengths with the same primer binding sequences. Since both templates are amplified with the same primer pair, identical thermodynamics and amplification efficiency are ensured. The amount of one of the templates must be known and, after amplification, products from both templates must be distinguishable. Different types of competitors have been used in cPCR, but in general, those competitors similar in size and base composition to the target work most effectively. RNA competitors should be used in quantitative reverse transcriptase (RT)-PCRs to address the problem of variable RT efficiency.

The yield of PCR product is described by the equation $Y = I(1 + e)^n$, where Y is the quantity of PCR product, I is the quantity of template at the beginning of the reaction, e is the efficiency of the reaction, and n is the number of cycles. In cPCR, this equation is written for both templates, as follows: competitor, $Y_c = I_c(1 + e)^n$; target, $Y_t = I_t(1 + e)^n$. Since e and n are the same for both the competitor and target, the relative product ratio Y_c/Y_t directly depends on their initial concentration ratio, I_c/I_t, and the function, $Y_c/Y_t = I_c/I_t$, is linear.

A single concentration of competitor is sufficient, in theory, to quantitate an unknown amount of target without the use of a standard curve. However, because analysis of two template species present in a sample at widely different amounts was imprecise in practice, cPCR using several concentrations of competitor within the expected concentration range of the target were generally performed. However, this approach provided no more accurate results than the use of a single concentration of competitor in a study of different approaches to standardization of cPCR (Haberhausen et al., 1998). The commercially available quantitative PCR for cytomegalovirus (CMV) and RT-PCR assays for HIV and hepatitis C virus (HCV) (Roche Molecular Systems, Pleasanton, CA) all use a single concentration of a competitor (quantitation standard) to determine the initial concentration of the target.

The commercially available quantitative PCR assays incorporate several technological innovations that have made

both qualitative and quantitative PCR feasible for clinical laboratories. The RT-PCR assays for quantitation of HIV-1 and HCV RNA use a single enzyme, a recombinant DNA polymerase from the bacterium *Thermus thermophilus*, to carry out both reverse transcription of the target RNA and amplification of the cDNA (Myers and Gelfand, 1991). Prior to the development of this polymerase, RT-PCR involved cumbersome two-enzyme systems. The assays incorporate dUTP and uracil-*N*-glycosylase for control of carryover contamination (Longo et al., 1990). A colorimetric microtiter plate system is used for detection of amplified products. In this system, biotinylated primers are used to amplify the target, and the biotin-containing PCR product is denatured and added to the microtiter well coated with specific capture probes. After hybridization with the capture probe, bound product is detected with a streptavidin-enzyme conjugate and a chromogenic substrate (Loeffelholz et al., 1992). This system for PCR product detection resembles an enzyme immunoassay and uses microtiter plate washers and readers commonly found in clinical laboratories. Finally, all of the manual quantitative PCR assays are being adapted for the Cobas system (Roche), which automates the amplification and detection steps.

A schematic of the Amplicor Monitor system (Roche) for the quantitation of HIV-1 RNA is shown in Fig. 1. After amplification, the samples are denatured, and aliquots are diluted in microtiter wells coated with probes specific for either the virus or the quantitation standard. After hybridization and color development, the ratio of HIV-1 RNA signal to that of the quantitation standard (QS) is used to calculate the number of HIV-1 RNA copies in the original sample using the following formula: virus copy number = (optical density × dilution factor)$_{Virus}$/(optical density × dilution factor)$_{QS}$ × input QS copies.

Real-Time PCR

In its simplest format, the PCR product is detected as it is produced using fluorescent dyes that preferentially bind to double-stranded DNA. SYBR green I is one such dye that has been used in this application (Morrison et al., 1998). In the unbound state, the fluorescence is relatively low, but when bound to double-stranded DNA, the fluorescence is greatly enhanced. The dye will bind to both the specific and nonspecific PCR products. The specificity of the detection can be improved through melting curve analysis. The specific amplified product will have a characteristic melting peak at its predicted melting temperature (T_m), whereas the primer dimers and other nonspecific products should have different melting temperatures (T_ms) or give broader peaks (Ririe et al., 1997).

The specificity of real-time PCR can also be increased by including hybridization probes in the reaction mixture. These probes are labeled with fluorescent dyes or with combinations of fluorescent and quencher dyes and rely on FRET to detect accumulating PCR product. FRET is a spectrophotometric process by which energy is transferred between molecules separated by 10 to 100 Å that have overlapping emission and absorption spectra. The theory behind this process was developed primarily by Förster (Clegg, 1992).

In the 5′ nuclease PCR assay (TaqMan), the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase is used to cleave a nonextendable hybridization probe during the primer extension

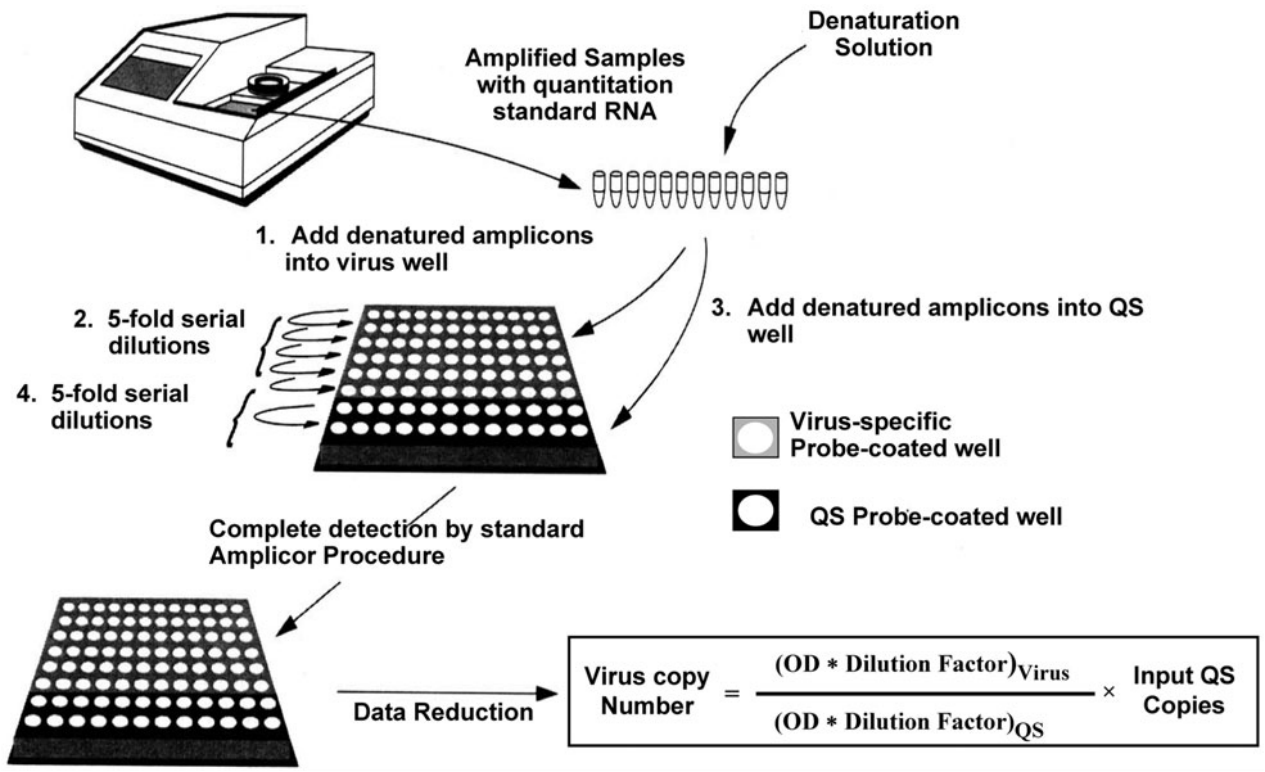

FIGURE 1 Schematic of the Roche Amplicor Monitor system for the quantitation of HIV-1 RNA. QS, quantitation standard; OD, optical density. (Provided by Roche Molecular Systems.)

phase of PCR (Holland et al., 1991). This approach uses dual-labeled fluorogenic hybridization probes. One fluorescent dye serves as a reporter, and its emission spectrum is quenched by the second fluorescent dye. The nuclease degradation of the hybridization probe releases the reporter dye, resulting in an increase in its peak fluorescent emission. The increase in fluorescent emission indicates that the specific PCR product has been made and the intensity of fluorescence is related to the amount of product (Heid et al., 1996). The 5′ nuclease probes are used commonly in assays designed for the ABI Prism sequence detection systems (Applied Biosystems, Foster City, CA).

Figure 2 shows a representative real-time PCR amplification plot and defines the terms used in real-time PCR quantitation. The amplification plot shows the normalized fluorescence signal from the reporter (R_n) at each cycle number. In the initial cycles of PCR, there is little change in the fluorescent signal. This defines the baseline for the plot. An increase above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The cycle threshold (C_T) is defined as the cycle number at which the fluorescence passes the fixed threshold. A plot of the log of the initial target concentration versus C_T for a set of standards is a straight line. Figure 3 shows a standard curve for a real-time PCR assay for CMV. The amount of target in an unknown sample is determined by measuring the sample C_T and using a standard curve to determine the starting copy number. The efficiency of real-time PCR amplification is calculated using the slope of the regression line of the standard curve with the equation efficiency = $10^{-\text{slope}} - 1$. In this case, the efficiency of amplification is 99.5%. Alternatively, the cycle number corresponding to the maximal change in fluorescence, the second derivative maximum, has a similar relationship to the initial target concentration.

A modification of the 5′ nuclease chemistry is used with minor groove binding (MGB) probes (Afonina et al., 2002). MGB probes replace the fluorescent quencher dye with nonfluorescent (dark) quencher and incorporate a small molecule that hyperstabilizes the probe-target duplex by folding into the minor groove of the double-stranded DNA. In the unbound state, the probe assumes a random coil configuration and the fluorophore is in close proximity to the dark quencher and emission from the fluorophore is efficiently quenched. This design allows the use of very short probes (12 to 17 nucleotides) because of the high T_m resulting from the interaction of the MGB with the DNA helix. These probes are particularly useful in assays where target sequence heterogeneity is a significant problem.

A pair of adjacent, fluorogenic hybridization probes are often used in assays designed for LightCycler (Roche), a rapid-cycle, capillary, microvolume fluorimeter and thermal cycler. These assays require two specially designed sequence-specific oligonucleotide probes that hybridize next to each other on the product molecule. The 3′ end of one probe is labeled with a donor dye, and the 5′ end of the other probe is labeled with an acceptor dye. The donor dye is excited by an external light source and, instead of emitting light, transfers its energy to the acceptor dye by FRET. The excited acceptor dye emits light at a longer wavelength than the unbound donor dye, and the intensity of the acceptor dye light emission is proportional to the amount of PCR product.

Real-time detection and quantitation of PCR product can also be accomplished using molecular beacons (Tyagi et al., 1998). Molecular beacons are hairpin-shaped oligonucleotide probes with an internally quenched fluorophore whose fluorescence is restored when the probes bind to a target nucleic acid. They are designed in such a way that the loop portion of the molecule is a probe sequence complementary to the target sequence. The stem is formed by the annealing of complementary arm sequences on the ends of the probe. A fluorescent dye is attached to one end of one arm, and a quenching molecule is attached to the end of the other arm. The stem keeps the fluorophore and quencher in close proximity, and no light emission occurs. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the stem and undergoes a conformational change that forces the stem apart and causes the fluorophore and quencher to move away from each other, restoring the fluorescence.

Real-time PCR methods decrease the time required to perform nucleic acid assays because there is no post-PCR processing time. Also, since amplification and detection occur in the same closed tube, these methods eliminate the

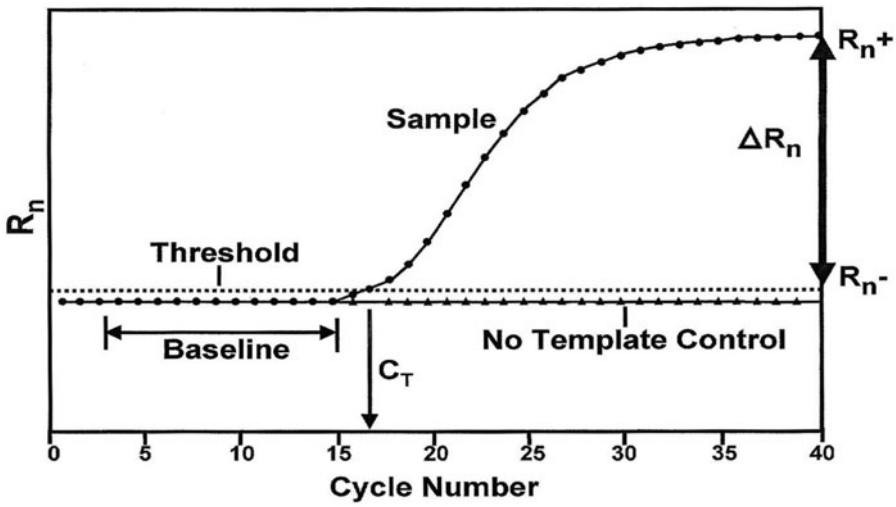

FIGURE 2 Real-time PCR amplification plot with commonly used terms and abbreviations. R_n, normalized fluorescent signal from reporter dye.

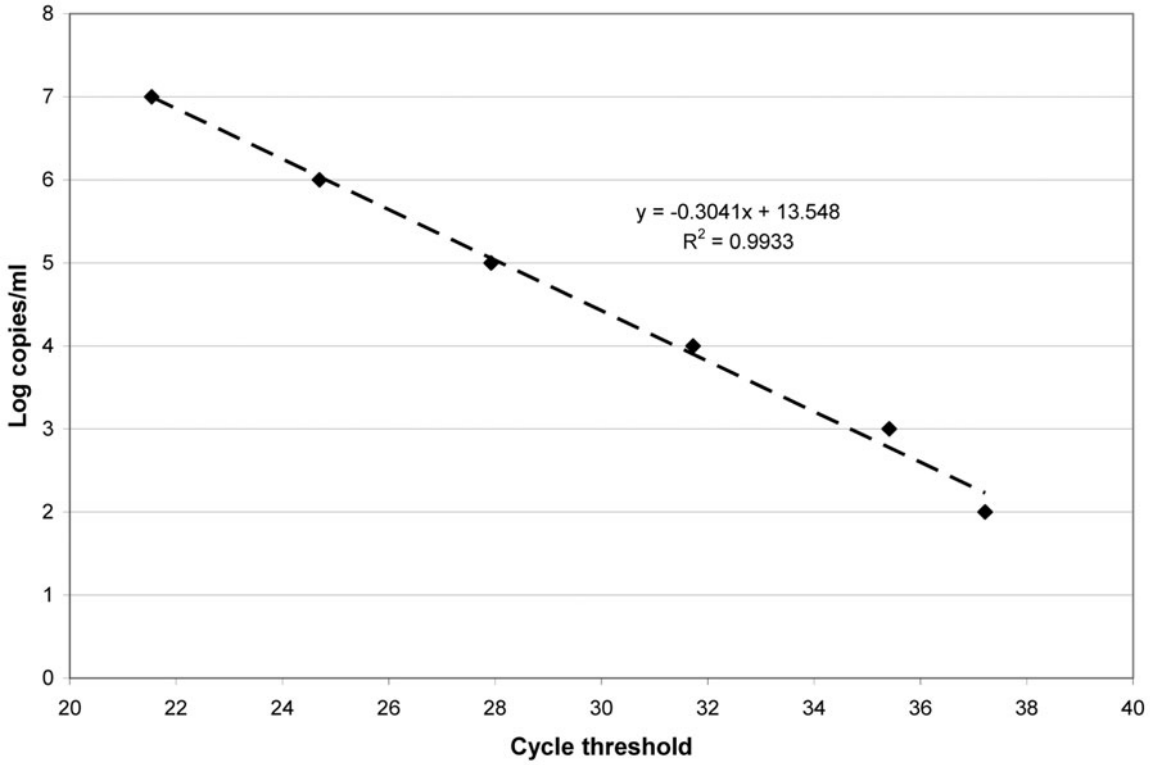

FIGURE 3 Example of a standard curve for a real-time PCR assay with the regression line equation and R^2 statistic. The efficiency of the PCR amplification can be estimated using the following equation: efficiency = $10^{-\text{slope}} - 1$.

postamplification manipulations that can lead to laboratory contamination with amplicon. Real-time PCR methods provide accurate and reproducible quantitation of target nucleic acids because analysis is performed early in the log phase of product accumulation and, as a result, are less prone to error resulting from differences in sample-to-sample amplification efficiency. These methods also have very large dynamic ranges of starting target molecule determination of at least 6 orders of magnitude.

A number of viral load assays based on real-time PCR methods have been developed in laboratories throughout the world, and an increasing number of analyte-specific reagents for several real-time PCR platforms are available commercially. The impact of this technology on the clinical microbiology laboratory has been the subject of recent reviews (Mackay, 2004; Espy et al., 2006). The next generation of FDA-cleared viral load assays for HIV and HCV will be based on real-time PCR methods (Barbeau et al., 2004; Braun et al., 2007).

NASBA

NASBA (bioMérieux, Durham, NC) is one of several transcription-based amplification methods that amplify RNA targets (Compton, 1991). In contrast to PCR, NASBA is an isothermal process that employs three enzymes, avian myeloblastosis virus RT, RNase H, and T7 RNA polymerase (Fig. 4). The amplification steps involve the formation of cDNAs from the target RNA with oligonucleotide primers containing a T7 RNA polymerase binding site. RNase H then degrades the initial strand of target RNA in the RNA-DNA hybrid after it has served as a template for the first primer. The second primer binds to the newly formed cDNA and is extended, resulting in the formation of double-stranded cDNAs with an intact T7 promoter. This DNA molecule serves as a substrate for the T7 RNA polymerase that transcribes multiple copies of antisense RNA. The antisense RNA molecules can also bind the second primer, and through the combined enzymatic activities, new double-stranded cDNA molecules are synthesized, which in turn serve as transcription templates for the RNA polymerase. In this way, exponential amplification of the targeted RNA sequence occurs.

The NucliSens HIV-1 QT assay for quantitation of HIV-1 RNA in plasma and other specimens is based on NASBA (Van Gemen et al., 1993). The nucleic acids are extracted from specimens using guanidinium thiocyanate and then purified by adherence to acidified silica. The extraction procedure is very robust and can be used to extract and purify nucleic acids from a variety of clinical specimens (Boom et al., 1990). An instrument that automates the sample preparation steps is available.

The primers in this assay target the *gag* gene region and amplify the target RNA as well as the three internal calibrator RNA molecules that are included in each reaction mixture. The internal calibrators are present at low, medium, and high copy numbers and are added to the sample prior to nucleic acid extraction to control for sample-to-sample variation of nucleic acid extraction and amplification. Each calibrator has a unique internal sequence that allows for discrimination of the calibrator and the target amplicons with specific probes that are labeled with ruthenium. The amplicons from the target and the three calibrators, Qa,

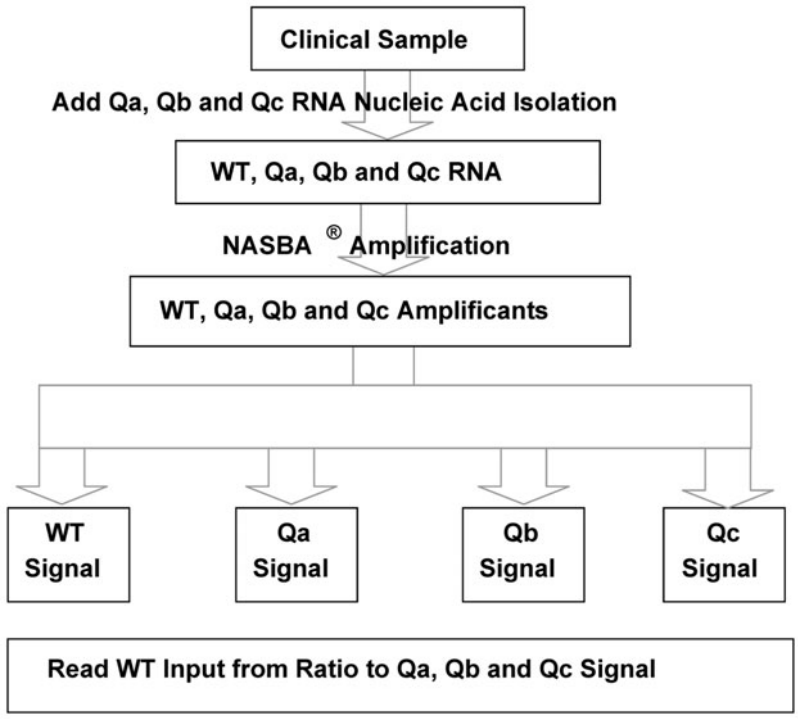

FIGURE 4 NASBA pathways for single-stranded RNA (A) and double-stranded DNA (B). RT, avian myeloblastosis virus RT; pol, T7 RNA polymerase; wavy lines, RNA; dashed lines, newly synthesized DNA; solid lines, primers. (Provided by Organon-Teknika.)

Qb, and Qc, are hybridized to capture oligonucleotides bound to magnetic beads and detected by hybridization to specific ruthenium-labeled probes in four separate microtubes (Fig. 5). The magnetic beads with the amplicon-probe complex are attached to the surface of an electrode with a magnet, and a voltage applied to this electrode triggers an electrochemiluminescent reaction. The light emitted by the ruthenium label is proportional to the amount of amplicon. The original starting concentration of the target is calculated from the relative amounts of the four amplicons.

bDNA

The bDNA signal amplification system (Siemens Medical Solutions Diagnostics, Malvern, PA) is a solid-phase, sandwich hybridization assay incorporating multiple sets of synthetic oligonucleotide probes and several simultaneous hybridization steps (Nolte, 1999). Multiple target-specific probes (5 to 9, depending upon the assay), termed capture extenders, are used to capture the target nucleic acid (DNA or RNA) onto the surface of a microtiter well plate (Fig. 6). A second set of target-specific probes (18 to 39, depending upon the assay), termed label extenders, hybridize to the target, and as in the first-generation assays, also serve as binding sites for the synthetic bDNA amplifier molecules. The amplifier molecules each have 15 identical arms, each of which can bind 3 alkaline phosphate-labeled probes. As many as 3,000 enzyme-labeled probes can be hybridized to each target molecule in this manner. Detection of the bound labeled probes is achieved by incubating the complex with an enzyme-triggerable chemiluminescent substrate, dioxetane, and measuring the light emission. Since the number of target molecules is not altered, the resulting signal is directly proportional to the concentration of the target nucleic acid. The quantity of the target in the sample is determined from a standard curve.

In the second- and third-generation bDNA assays, a preamplifier molecule is used to further increase the number of labeled probes that can be bound to the target (Fig. 6). The label-extender probes are designed such that two probes must be bound to adjacent regions of the target for efficient hybridization to the preamplifier molecule to occur. Each preamplifier molecule can bind multiple amplifier molecules. In the second-generation bDNA assay for HIV-1 RNA, each captured RNA molecule may bind as many as 10,080 separate alkaline phosphatase-labeled probes (Kern et al., 1996). The lowest concentration of HIV-1 RNA that could be distinguished from the negative control was 390 copies/ml in this assay.

The first and second generations of the bDNA assays were limited by nonspecific hybridization between the amplification probes and other nucleic acids. Short regions of hybridization between any of the probes comprising the amplification system (preamplifier, amplifier, and labeled probe) and any nontarget nucleic acid sequence leads to amplification of the background signal. Capture probes, capture extenders, and sample nucleic acids are all sources of this background hybridization (Collins et al., 1997).

To reduce the hybridization potential to all nontarget nucleic acids, the nonnatural bases, isocytidine (isoC) and isoguanosine (isoG), were incorporated into the amplification probes in the third-generation assays. isoC and isoG are among the 6 base pairs capable of forming Watson and Crick base pairs joined by mutually exclusive hydrogen-bonding schemes incorporating three hydrogen bonds (Piccirilli et al.,

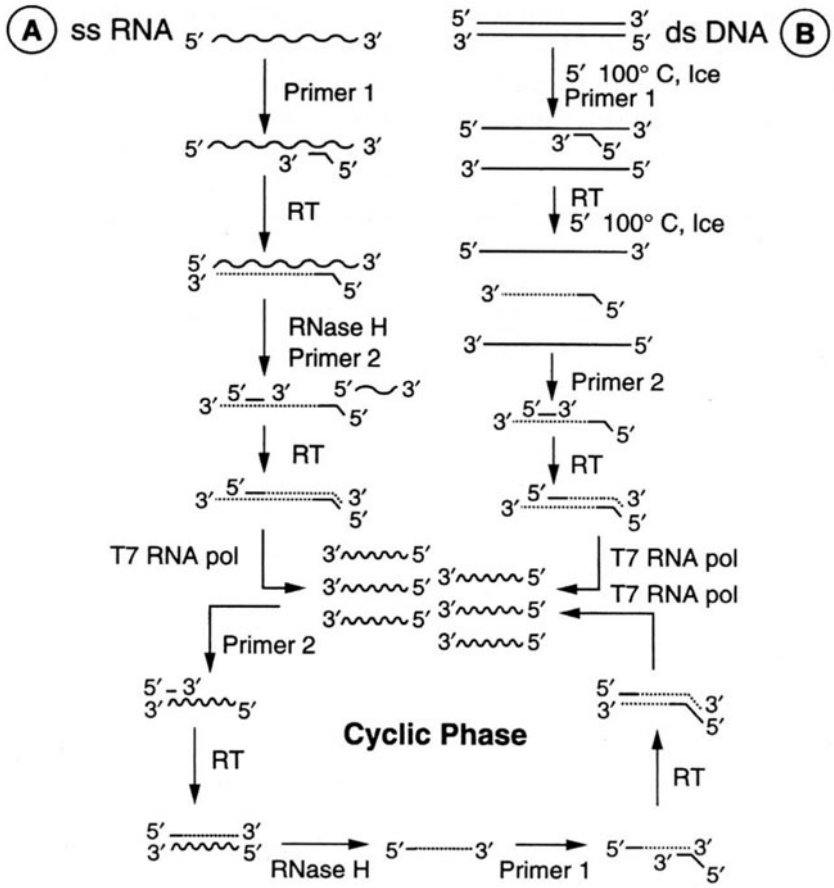

FIGURE 5 Principle of viral nucleic acid quantitation with NASBA. WT, wild-type sequence; Q, calibrator; ECL, electrochemiluminescence. (Provided by Organon-Teknika.)

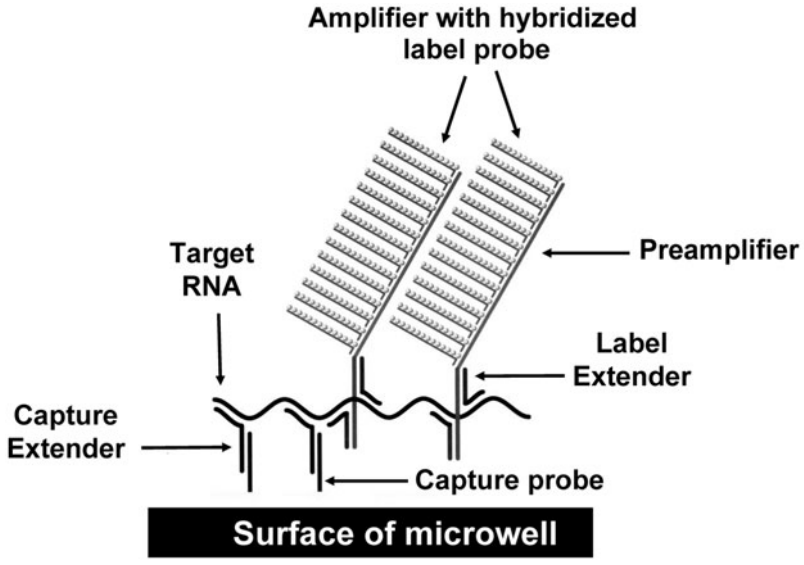

FIGURE 6 Diagram of a third-generation bDNA assay. (Provided by Siemens.)

1990; Switzer et al., 1993). Although isoC and isoG can be incorporated into duplex DNA and RNA by DNA and RNA polymerases, respectively, these potential extra letters in the genetic alphabet are not found in natural oligonucleotides. IsoC and isoG bases pair with each other but not with any of the four naturally occurring bases. Sequences containing isoC-isoG base pairs are –2°C more stable per base than their C-G congeners. The use of isoC- and isoG-containing probes in the system bDNA assays increased the target-specific amplification without a concomitant increase in the background from nontarget sequences, thereby greatly enhancing the sensitivity to a detection limit of approximately 50 molecules/ml.

The System 340 platform automates incubation, washing, reading, data processing, and report generation. The automation dramatically reduces operator-to-operator differences while decreasing labor requirements. Future improvements in reproducibility and operational efficiency await the development of a system for automated sample preparation.

HCA

Hybrid capture assays (HCAs) employ a signal amplification technology that can be applied to the detection and quantitation of DNA or RNA target molecules without amplification (Cope et al., 1997c; Mazzulli et al., 1999). This technology depends upon the formation of DNA-RNA hybrid molecules and uses antibodies specific for these hybrids to capture and detect them. A large, single-stranded RNA probe is added to the specimen containing target DNA. The DNA-RNA hybrids are captured by antibodies specific for these hybrids coated on a solid support. Bound hybrids are then reacted with alkaline phosphatase-conjugated antibodies specific for DNA-RNA hybrids. The enzyme-conjugated antibodies are then detected by addition of a chemiluminescent substrate (Fig. 7). Because each DNA-RNA hybrid binds approximately 1,000 antibody conjugate molecules, each of which is labeled with 3 alkaline phosphatase molecules, the resulting signal is amplified at least 3,000-fold. The light output is measured with a luminometer, and the intensity of the emitted light is proportional to the concentration of target DNA in the specimen. An HCA for the qualitative detection of CMV DNA (Digene, Gaithersburg, MD) was cleared by the FDA. For quantitative determinations, a series of standards are run in parallel, allowing the generation of a standard curve that is used to quantitate the amount of target nucleic acid in the specimen (Mazzulli et al., 1996; Ho et al., 2000).

Key Assay Performance Issues

There a number of key performance characteristics that affect the results, use, and interpretation of quantitative nucleic acid assays (Dailey and Hayden, 1999; Shepley and Wolk, 2004). All of these characteristics should be considered when comparing different assays and technologies.

Linearity

The extent to which assay results are proportional to the concentration of analyte is termed assay linearity. Linearity

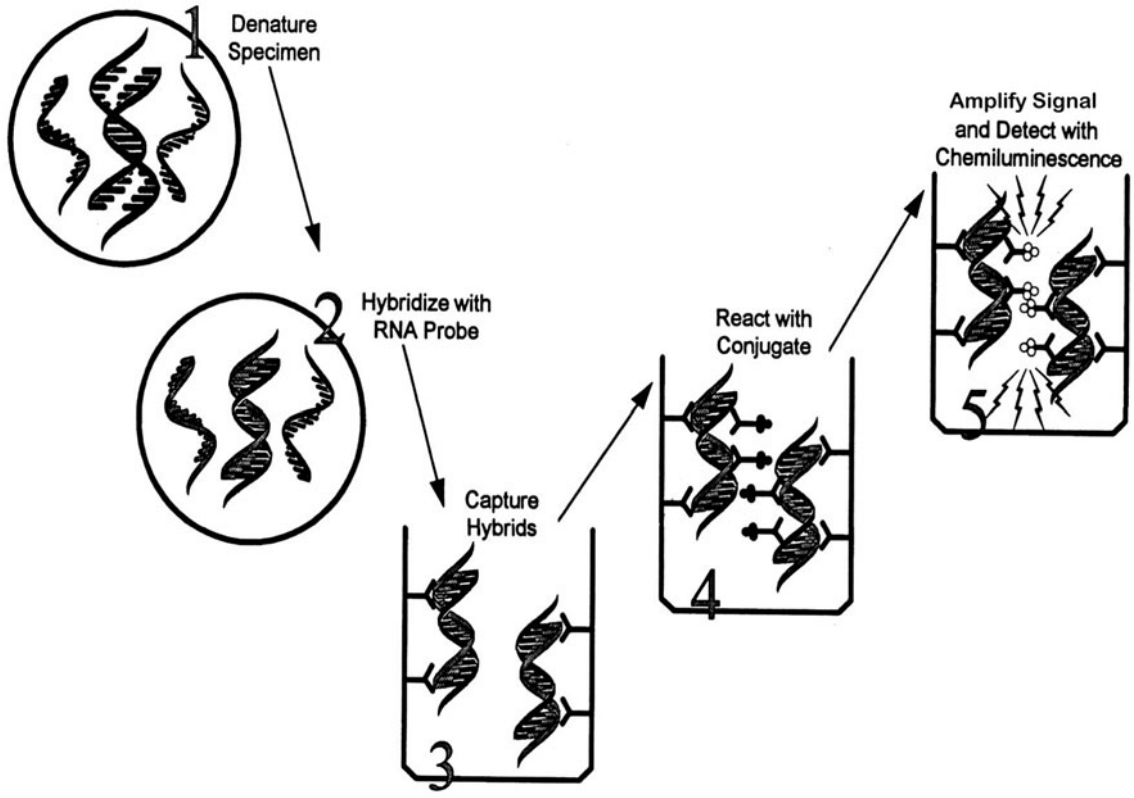

FIGURE 7 Diagram of an HCA. The specimen is denatured (1) and then hybridized with an RNA probe (2). If the target nucleic acid is present, the resulting DNA-RNA hybrid is captured by an antibody specific for the hybrids (3). Multiple alkaline-phosphatase-conjugated antibodies bind to the captured hybrids (4). A chemiluminescent substrate emits light that is measured in a luminometer (5). (Provided by Digene.)

is determined by testing samples containing multiple levels of analyte and plotting the assay results against the expected results. The extent to which this plot approximates a straight line is a measure of the assay linearity. The linear range of an assay is the concentration range over which the assay quantitates with acceptable accuracy, precision, and linearity. The linear range should include levels of viral load commonly seen in patients.

Accuracy

The accuracy of an assay is the extent to which it agrees with the true value or an accepted reference standard. Unfortunately, accepted reference standards for viral nucleic acids are not widely available. One important exception is HCV RNA. A World Health Organization international standard for nucleic acid amplification assays has been established (Saldanha et al., 1999). The standard has been assigned a value of 10^5 IU/ml and is available from the National Institute for Biological Standards and Control, South Mims, United Kingdom, and has been widely accepted. An HIV-1 international standard has also been developed but not widely accepted by the diagnostic community (Holmes et al., 2001).

The calibrators used in different quantitative nucleic acid assays are characterized using different techniques, and as a result, the number of virus copies reported by one assay may not agree with that reported by another assay. The availability of accepted reference standards should help establish the accuracies of the various assays and permit more comparison among methods. Because different viral load assays currently use different standards, the same assay should be used for longitudinal monitoring of patients. If clinical decisions are based on specific viral load levels published in the literature, remember that those numbers may only apply to the particular assay used in that study.

Agreement

In the absence of accepted reference standards, the similarity or equivalence of results obtained by different methods is often a key performance issue when laboratories change methods. A plot of the difference between methods against their means is more informative than a plot of the results of one method against those of another (Bland and Altman, 1986). This plot will show any relationship between the measurement error and the true value. Since the true value is often not known, the mean of the two methods is the best estimate of the true value. Agreement between assay results is best assessed by determining the mean difference and the standard deviation of the differences. Two standard deviations around the mean difference define the limits of agreement between the assays.

Precision

Agreement between replicate measurements on the same sample is termed precision. Precision is a gauge of the variability inherent in an assay due to error or noise in the assay. The precisions of viral load assays have been reported using a variety of different statistical measures, including standard deviation, coefficient of variation, range, and relative difference (Dailey and Hayden, 1999). Regardless of which measure is used, viral load assay variability is best analyzed after $\log_{10}$ transformation of the data. Log transformation of viral load data is important because viral replication is a multiplicative, not an additive, process. In addition, the parametric statistical tests often used to describe and analyze viral load data cannot be used with untransformed data because, without log transformation, the data are not normally distributed and variability is not homogenous (Dailey and Hayden, 1999).

Tolerance Limit

The tolerance limit of an assay is the difference between two sequential patient samples that can be considered significantly different within a certain confidence interval, usually 95% (Patel, 1986). The tolerance limit should be a function of both the biological variability of viral load and the precision of the assay. For example, the tolerance limit for HIV-1 RNA assays is generally accepted to be 0.5 $\log_{10}$ copies of HIV-1 RNA. As a result, only changes greater than 0.5 $\log_{10}$ (3.2-fold) in HIV-1 viral load are considered significant.

Lower Limit of Quantitation

The lower limit of quantitation is the lowest concentration of analyte that can be quantitated with acceptable accuracy and precision. Viral load assays tend to be less precise at or near the limit of quantitation. As a result, the significance of changes in viral load at the lowest levels of quantitation should be evaluated carefully.

The limit of detection is the lowest concentration of analyte that can be discriminated for background but not necessarily quantitated accurately. Limit of detection and analytical sensitivity are terms that are used interchangeably. A viral load assay may accurately quantitate levels as low as 500 copies/ml but may detect samples as positive with as few as 50 copies/ml.

Specificity

The specificity of an assay is the extent to which it measures only the analyte it was intended to measure. The specificities of viral load assays are evaluated by testing specimens from patients without evidence of infection with that virus, usually seronegative individuals. Viral load assays are typically used to evaluate patients for whom the diagnosis is already established. These tests have not been designed as diagnostic tests and have not been tested extensively on seronegative populations. The specificity of HIV-1 RNA assays ranges from 95 to 98% (Dailey and Hayden, 1999). False positives in signal amplification assays can result from samples with high background, and in target amplification assays, they can result from amplicon product contamination. Inappropriate use of these assays has led to misdiagnoses (Rich et al., 1999).

Subtype Genetic Variation

A key component of all viral load assays is the hybridization of oligonucleotide probes or primers to the target nucleic acid. Two of the important targets for viral load assays, HIV-1 and HCV, are RNA viruses that display impressive genetic heterogeneity. Probe and primer design must take into account the inherent genetic variability of the target if the assay is to provide equal quantitation of all subtypes (Kwok et al., 1990). Primer mismatches at critical positions in the target may not completely prevent amplification in a PCR assay, but they can substantially reduce the efficiency of amplification, leading to results that are much lower than anticipated (Vandamme et al., 1996; Dunne and Crowe, 1997; Hawkins et al., 1997).

APPLICATIONS

HIV-1

When HIV-1 enters a new host, there is typically a burst of viremia which is then controlled by the onset of immune responses. The subsequent level of plasma virus is a reflection

of the equilibrium reached between the virus and the host after the initial battle and is generally maintained for years. This steady-state level varies from individual to individual and is predictive of long-term clinical outcome in the absence of effective antiretroviral therapy. This was first demonstrated in 1996 as part of a multicenter AIDS cohort study (Mellors et al., 1996). In this study, investigators measured the viral load in stored plasma samples collected from 180 untreated HIV-1-infected men and traced the fate of those patients. The risk of AIDS and death in study subjects was directly related to viral load at study entry. Plasma viral load was a better predictor of progression to AIDS and death than the number of $CD4^+$ lymphocytes. These results established that viral load critically influenced disease progression and suggested that lowering viral levels as much as possible and as early as possible with therapy may be essential to prolonging life. Subsequent studies both confirmed that viral load influences disease progression and changed how new therapies are evaluated (Coombs et al., 1996; Katzenstein et al., 1996; O'Brien et al., 1996).

In addition to determining prognosis, viral load data are also useful in monitoring therapeutic response in patients receiving antiretroviral therapy. Multiple analyses of more than 5,000 patients who participated in 18 clinical drug trials with viral load monitoring demonstrated a reproducible dose-response association between decreases in the magnitude of plasma viremia and improved clinical outcome based on standard endpoints of new AIDS-defining diagnoses and survival (Centers for Disease Control and Prevention, 1998a, 1998b). This relationship occurred over a range of patient baseline characteristics, including pretreatment viral load, $CD4^+$ cell count, and prior antiretroviral drug experience.

Viral load testing is an essential parameter in guiding decisions to begin or change antiretroviral therapy (Hammer et al., 2006). The aim of antiretroviral therapy is the maintenance of the plasma HIV-1 RNA level below the limits of detection of the most sensitive assays commercially available (i.e., <50 copies/ml). Effective regimens in compliant patients result in a decrease of at least 1 $\log_{10}$ copies/ml per month, and suppression of plasma viral load to <50 copies/ml will usually be attained by 16 to 24 weeks, depending upon the initial pretreatment HIV-1 RNA level. After 48 weeks, viral load tests should be obtained at regular intervals (e.g., every 3 to 4 months) to confirm that the HIV-1 RNA level remains below the limit of detection. Rebound of viral load after reaching undetectable levels should prompt a careful evaluation of the patient's adherence to the drug regimen. Isolated low-level rebounds to levels of 50 to 500 copies/ml are often of no clinical consequence; however, consecutive rebounds to >500 copies/ml can be associated with development of drug resistance mutations and virologic failure.

HCV

Quantitative determinations of HCV RNA in serum have been used to increase our understanding of the natural history of chronic HCV infection, predict which patients are likely to respond to therapy, and determine whether a virologic response to therapy has occurred. HCV viral load is relatively stable without significant fluctuation in untreated patients with chronic hepatitis C (Nguyen et al., 1996). However, unlike in HIV-1 infection, the HCV load does not correlate with severity of disease or prognosis (National Institutes of Health, 2002). High HCV load is an important risk factor in mother-to-child transmission of the virus. Although the overall risk of transmission of HCV from infected mother to child is low (approximately 5%), the risk of transmission increases with high levels ($\geq 10^6$ copies/ml) of maternal viremia (Ohto et al., 1994).

Rates of response to a course of standard therapy with interferon and ribavirin are higher in patients with low levels of HCV RNA (Davis et al., 1998; McHutchinson et al., 1998; Poynard et al., 1998). Low-level HCV viremia is usually defined as $< 2 \times 10^6$ copies/ml (800,000 IU/ml). Although viral load is an independent predictor of response to therapy, viral genotype is a stronger predictor than viral load, and the level of viremia should not be used as a reason to deny treatment (Lok and Gunaratnam, 1997; National Institutes of Health, 2002).

Response to therapy in hepatitis C is defined biochemically as the normalization of alanine aminotransferase levels and virologically as absence of detectable HCV RNA in the blood. Typically, end-of-treatment responses are assessed with a sensitive qualitative rather than quantitative assay. However, quantitative tests can be used in early assessments of treatment response. For patients with genotype 1 infection who have a <2 log drop in HCV RNA levels after 12 weeks of therapy, it is unlikely that they will have a sustained virological response (Manns et al., 2001; Fried et al., 2002). Similarly, patients with genotype 2 or 3 infections who have an undetectable HCV RNA level after 4 weeks of therapy may receive 12 weeks of therapy without any compromise in efficacy compared to the standard 24-week course (Mangia et al., 2005). Early cessation of therapy spares patients the higher cost and toxicity associated with longer courses.

HBV

Approximately 5% of individuals infected with hepatitis B virus (HBV) become chronic carriers of the HBV surface antigen (HBsAg). Chronic HBsAg carriers can be divided into two groups: those with low-level viral replication and normal liver function tests and those with active viral replication and progressive liver disease. The secretory version of the HBV core protein, the e antigen (HBeAg), has traditionally served as a marker for active viral replication. However, the level of HBV DNA in serum or plasma better reflects the replicative activity of HBV than the presence or absence of HBeAg.

Determining levels of HBV DNA in serum is useful for identifying individuals most likely to respond to antiviral therapy and evaluating the response to therapy (Zoulim et al., 1992; Lok et al., 2001). During therapy, the clinician can assess its efficacy by measuring either the absolute reduction or the kinetics of decrease in viral load.

CMV

CMV viral load determinations have been used to predict the subsequent development or relapse of CMV disease before the onset of symptoms in solid-organ transplant recipients, bone marrow transplant recipients, and AIDS patients. There is substantial evidence that weekly monitoring of CMV load during the first 3 months after transplant is useful to predict CMV disease in solid-organ transplant recipients (Cope et al., 1997a; Cope et al., 1997b; Toyoda et al., 1997). However, the breakpoints for DNA copy number that predict the development of CMV disease in different patient populations are not well defined. Available data on DNA copy numbers from different laboratories are difficult to compare because of differences in the methods used to quantitate CMV DNA (Boeckh and Boivin, 1998).

There is only a moderate association between high systemic viral load with CMV disease in allogeneic bone marrow

transplant recipients (Zaia et al., 1997; Gor et al., 1998). A significant portion of patients may develop CMV disease with low viral load or progress rapidly from low DNA levels to overt disease. Severe acute graft-versus-host disease requiring treatment with steroids is the clinical setting where rapid progression to CMV disease may occur. Quantitative molecular methods are probably not needed to monitor CMV seropositive autograft recipients due to the low incidence of CMV disease in these patients.

In HIV-1-infected individuals, there is an association between a high CMV load and CMV disease (Rasmussen et al., 1995; Boivin et al., 1997). CMV load also predicts the development of CMV disease (Boivin et al., 1996; Shinkai et al., 1997) and response to antiviral therapy (Spector et al., 1996). In addition, a high CMV load is an independent predictor of poor survival in most studies (Spector et al., 1998). A variety of samples, including whole blood, plasma, and leukocytes, and a variety of molecular methods have been used for viral load determinations, and it is not known which sample or method is optimal.

A decline in systemic CMV load occurs after initiation of effective antiviral therapy in solid-organ transplant recipients, bone marrow transplant recipients, and HIV-1 infected individuals (Boeckh and Boivin, 1998). Viral load testing may also be used to monitor the emergence of drug-resistant CMV strains and the progression of CMV disease (Boivin et al., 1996; Smith et al., 1996; Boivin et al., 1997).

There also has been considerable interest in using quantitative nucleic acid tests to assess CMV load in the central nervous system, lungs, and eyes. CMV quantitation has been used to study the viral load in cerebrospinal fluid in the different central nervous system manifestations of CMV infection in AIDS patients. Patients with CMV polyradiculopathy have higher viral loads in cerebrospinal fluid than patients with CMV encephalitis (Shinkai et al., 1997). AIDS patients with CMV encephalitis have a 10- to 1,000-fold-higher CMV load in autopsy brain specimens than did patients without histologic evidence of encephalitis (Kuhn et al., 1995). Quantitative nucleic acid tests can distinguish asymptomatic pulmonary shedding of CMV from CMV pneumonia in immunocompromised patients (Boivin et al., 1996). Testing for CMV DNA in the eyes may provide a virologic measurement of the response to therapy in patients with low or undetectable systemic viral load and may provide a means to study the pathogenesis of CMV retinitis. The aqueous humor provides an accessible sample for DNA quantitation and may reflect viral load in the eyes of AIDS patients (Gerna et al., 1994; Mitchell, 1996).

EBV

Epstein-Barr virus (EBV) load may be a useful prognostic marker for development of EBV-related posttransplant lymphoproliferative disorders (PTLD) and may be useful for monitoring the effects of appropriate interventions (Rowe et al., 1997; van Esser et al., 2001; van Esser et al., 2002). EBV-related PTLD occur in 1 to 10% of transplant recipients and involve expansions of B or T cells ranging from reactive hyperplasias to large-cell lymphomas. PTLD can be rapidly fatal if not accurately diagnosed and often respond to decreased immunosuppression and reconstitution of the immune system. There is no agreement on whether leukocytes, whole blood, serum, or plasma is used for monitoring patients for EBV lymphoproliferative disease. Leukocytes and whole blood give higher sensitivity for detection of EBV DNA, while serum and plasma give higher specificity for EBV-associated disease.

Other Herpesviruses

Quantitative nucleic acid assays may also help establish disease associations for other ubiquitous herpesviruses including human herpesvirus 6 (HHV-6), HHV-7, and HHV-8. Similar to CMV, the high rate of reactivation and asymptomatic excretion of these viruses make it difficult to relate infection to disease without using viral load as a measure of active virus replication.

BKV

BK virus (BKV) is a member of family *Polyomaviridae*, which also includes JC virus and simian virus 40. The seroprevalence of BKV reaches nearly 100% in early childhood, generally after an asymptomatic primary infection, and declines to 60% to 80% in adulthood. After primary infection, the virus can remain latent in many sites, most notably the epithelium of the urinary tract, until an immunosuppressed state allows reactivation and replication of the virus. Replication of BKV in immunocompromised hosts may be asymptomatic or cause organ dysfunction. The kidney, lung, eye, liver, and brain are sites of BKV-associated disease. BKV disease in the urinary system manifests as hemorrhagic or nonhemorrhagic cystitis, ureteric stenosis, or tubulointerstitial nephritis (Reploeg et al., 2001; Hirsch and Steiger, 2003).

Although BKV was first isolated from the urine of a renal transplant recipient in 1971 (Gardner et al., 1971), the association between nephropathy and the presence of BKV in renal transplant recipients was not reported until 1995 (Purighalla et al., 1995). BKV replication in renal allografts can lead to progressive graft dysfunction and, potentially, graft failure. The recognition of BKV-associated nephropathy (BKVN) in renal transplant recipients coincided with the use of newer immunosuppressive drugs such as tacrolimus, sirolimus, and mycophenolate mofetil; however, the risk factors for development of BKVN have not been elucidated (Hirsch et al., 2005). The prevalence of BKVN ranges from 1 to 10% in kidney transplant recipients, with loss of allograft function in about one-third to one-half of these cases (Hirsch et al., 2005).

The signs and symptoms of BKVN are mild and nonspecific, often with only a gradual increase in serum creatinine levels over weeks as the allograft loses function (Ramos et al., 2002). A definitive diagnosis of BKVN is obtained through histopathology of the biopsied kidney; the characteristic BKVN pattern includes viral cytopathic changes in epithelial cells and interstitial inflammation and fibrosis. However, these changes are not pathognomonic for BKVN, and most centers use immunohistochemical staining with antibodies specific for polyomavirus proteins to confirm the diagnosis (Hirsch et al., 2005). Because of the focal nature of the nephropathy and the possibility of sampling error, negative biopsy results do not rule out BKVN. In addition, biopsy of the kidney is an invasive procedure that is impractical for serial monitoring, early diagnosis, and clinical management of patients with BKVN.

Other less-invasive diagnostic methods for BKVN have also been assessed. Urine cytology may reveal renal epithelial cells with intranuclear viral inclusion bodies, termed decoy cells (Drachenberg et al., 2001), although the positive predictive value for BKVN is low. Quantification of BKV DNA or mRNA in urine by nucleic acid amplification methods has been proposed as a method to monitor changes in BKV replication (Ramos et al., 2002; Randhawa et al., 2004; Bressollette-Bodin et al., 2005). However, physiological changes of urine constituents and use of different urine fractions may give rise to considerable variation in viral load

values that may complicate the identification of diagnostic thresholds (Hirsch and Steiger, 2003). PCR methods for detection and quantitation of BK viremia are used in the diagnosis and management of BKVN because viremia precedes development of nephropathy in all cases and viremia has a higher positive predictive value for BKVN than viruria (Compton, 1991; Hirsch et al., 2002). An interdisciplinary analysis and recommendations for diagnosis and management of BKVN were published in 2005 (Hirsch et al., 2005).

Advantages and Disadvantages

Prior to the development of quantitative molecular techniques, laboratories were limited to cumbersome culture methods or insensitive antigen detection methods to determine viral load. The molecular methods are more rapid and cost-effective than conventional virological methods and have created new opportunities for the clinical laboratory to impact patient care. The molecular approach is the only approach available for detection and quantitation of medically important viruses like HCV and the only practical approach for quantitation of the other medically important viruses.

Each of the quantitative molecular techniques has particular strengths and limitations that are inherent in the underlying nucleic acid amplification strategies. The major commercial viral load assays employ either target or signal amplification. Target amplification systems typically employ a single pair of primers, each of which is usually 20 to 40 bases in length. Mismatches between the primer and target sequence can lead to failure to amplify the target or to inefficient amplification, depending on the number and position of the mismatches. Primers must be carefully selected from highly conserved regions to ensure equal amplification of all genotypes. Signal amplification assays either employ numerous probes or large probes that cover larger regions of the target genome. In practice, signal amplification assays are less prone to errors resulting from target sequence heterogeneity.

Target amplification methods are more sensitive than signal amplification methods, with limits of detection in the 20- to 50-copy/ml range for the most sensitive PCR-based assays. These methods achieve this level of sensitivity by producing billions of copies of target nucleic acid. A major challenge in developing quantitative target amplification assays has been in establishing the relationship between the initial amount of the target sequence in the specimens and the amount of amplified product. As a result, target amplification methods tend to be less precise than signal amplification methods. The number of target molecules is not altered with signal amplification methods. Therefore, the amount of signal is directly proportional to the amount of target sequence present in the clinical sample.

Target amplification methods are enzymatic processes that are prone to sample inhibition, and, as a consequence, employ multistep sample preparation protocols to partially purify nucleic acids. Sample preparation is less cumbersome for signal amplification assays, since they are less prone to sample inhibition.

False-positive reactions are concerns with both target and signal amplification assays but for very different reasons. The tremendous numbers of product molecules produced in target amplification assays can be difficult to contain. Physical separation of pre- and postamplification activities, unidirectional workflow, plugged pipette tips, physical containment, and enzymatic and chemical methods for amplicon inactivation are all used to limit occurrence of false positives due to amplicon cross-contamination with conventional PCR. Since amplification and detection occur in the same reaction vessel with real-time PCR methods, amplicon cross-contamination is not an issue. False positives occur in signal amplification assays due to sample matrix effects that can lead to high background counts. The reported false-positive rates for the various bDNA assays are 2 to 5%.

Tips

The commercially available viral load assays each have their particular strengths and limitations. No single viral load assay is ideal for every laboratory setting. When choosing among the available assays, many issues should be considered, including the type of patients and specimens to be tested, available work space, technical skills of laboratory staff, work flow, volume of testing, and analysis time. Table 1 compares the features of the major commercially available quantitative HIV-1 RNA assays.

FUTURE

Viral load testing in HIV-1, HCV, HBV, CMV, EBV, and BKV infections are currently standards of care. In the future, viral load testing will be driven by the development of antiviral agents for other chronic viral infections and by the need for diagnostic tools that distinguish active from latent viral infections in immunocompromised patients.

On the technology front, there will be increased use of automation in viral load assays. Significant progress has already been made in automating the amplification and detection steps. Sample preparation remains the greatest challenge for automation, but completely automated and integrated solutions are becoming increasingly available to clinical laboratories. The next generation of FDA-cleared amplification assays will combine amplification and detection in a single closed vessel with automated sample processing. These assays will be less technically demanding and faster.

TABLE 1 FDA-cleared viral load assays[a]

Virus	Manufacturer	Test name	Method
HBV	Roche Molecular Diagnostics, Pleasanton, CA	Cobas TaqMan HBV test	PCR
HCV	Siemens Medical Solutions Diagnostics, Malvern, PA	Versant HCV RNA 3.0 assay	bDNA
HIV-1	Siemens Medical Solutions	Versant HIV-1 RNA 3.0 assay	bDNA
	bioMérieux, Inc., Durham, NC	NucliSens HIV-1 QT	NASBA
	Roche Molecular Diagnostics	Cobas Amplicor HIV-1 test v1.5	RT-PCR
		Cobas AmpliPrep/Cobas TaqMan HIV-1 test	Real-time RT-PCR
	Abbott Molecular, Des Plaines, IL	Real-time HIV-1	Real-time RT-PCR

[a]Information was current as of October 2008.

These methods will also eliminate many of the concerns about false-positive results due to amplicon cross-contamination.

CONCLUSIONS

Viral load assays have created new opportunities for the clinical laboratory to impact patient care. The clinical applications for these assays are increasing and are becoming more widely available. A thorough understanding of the key performance issues and features of the available quantitative molecular techniques is essential to the practice of modern clinical virology.

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Flow Cytometry

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16

Virus-cell interactions have been analyzed for more than 35 years by using fluorochrome-labeled antibodies in conjunction with flow cytometry. In vitro studies of virus-cell interactions include the detection and quantification of (i) the binding of fluorochrome-labeled viruses to their receptors on cell surfaces; (ii) fluorochrome-labeled viral antigens on the cell surface, in the cytoplasm, and in the nuclei of virus-infected cells; (iii) virus-induced apoptosis; and (iv) the effects of virus infection on the expression of cellular antigens and nucleic acid synthesis. In vivo studies have used fluorochrome-labeled monoclonal antibodies and flow cytometry to detect and quantify virus-infected cells directly in clinical specimens. The published literature before 2000 has been reviewed (McSharry, 2000a, 2000b, 2000c). Since that time, the number of publications reporting on the use of flow cytometry in virology has exploded to the point that a review of the extant literature is beyond the scope of this chapter. Therefore, this chapter will not be inclusive but will cover the following subjects: (i) a brief history of flow cytometry; (ii) a definition of flow cytometry; (iii) a description of the mechanics of a flow cytometer; (iv) the use of flow cytometry in detecting and quantifying human cytomegalovirus (hCMV)-infected tissue culture cells; (v) the use of flow cytometry for studying apoptosis of virus-infected cells; (vi) the use of flow cytometry for measuring the effect of virus infection on the cell cycle; and (vii) the use of flow cytometry in drug susceptibility testing.

HISTORY

The fluorescence-activated cell sorter (FACS) was developed in the early 1970s to study isolated populations of viable cells for immunology. The original instruments were large, contained powerful dual lasers that were water cooled, and were able to analyze and sort cells. In the modern era (1990 to the present), the flow cytometer has been simplified to a more compact, air-cooled, single or double laser, analytical instrument for use in immunology, cell biology, molecular biology, pathology, and diagnostic microbiology. Flow cytometry has usually been used to examine cells; however, a flow cytometer has recently been described that counts virus particles (Stoffel et al., 2005). Thus, in the future, flow cytometric studies in virology will include the study of virus particles as well as the study of virus-infected cells. For a detailed history of flow cytometry, see Shapiro (1995, 2004).

FLOW CYTOMETRY

Definition of Flow Cytometry

Flow cytometry can be defined as the measurement of the physical and/or chemical characteristics of cells while they pass single file in a fluid stream through a measuring apparatus (Watson, 1991; Shapiro, 1995; Robinson et al., 1999). Simple flow cytometers use a single argon ion laser to simultaneously measure the light scatter properties of cells and any fluorescence associated with the cells. The light scatter properties of each cell are measured when cells pass through the laser beam and scatter light. The scattered light passes through lenses that separate it into forward angle light scatter (FW-SC) and right angle light scatter (RT-SC). The light scatter properties of cells are used to count cells and determine their size and granularity. If the cells passing through the laser beam are labeled with one or more fluorochromes directed against specific cellular and/or viral components, the laser light excites the fluorochrome(s), causing each fluorochrome to emit light at a higher wavelength. The emitted light from light scatter and/or fluorochromes is separated into individual components by lenses, captured by photomultiplier tubes, and digitized, and the data are displayed on a monitor screen, printed on paper to yield a hard copy, and stored in a computer for a permanent file that can be used for further data analysis at a future time. Thus, the flow cytometric analysis of fluorochrome-labeled cells yields information on the number, size, and granularity of the cells, the number of fluorochrome-labeled cells, and the amount of fluorochrome associated with each cell. The simultaneous measurement of a number of physical and biochemical characteristics of each cell is known as a multiparametric analysis. The ability of flow cytometry to perform multiparametric analyses gives this technology the analytical power to identify populations of cells (phenotypes) that are undergoing particular biological events (apoptosis, DNA replication, viral infection, etc.). The analysis of the light scatter properties of cells is often used to separate different cell populations in a sample containing cells of different sizes and complexity, such as peripheral blood mononuclear cells. Fluorochrome-labeled antibodies to cellular and/or viral antigens and nucleic acids are used to further identify cells within each of the separated populations. Several thousand cells can be analyzed per second, yielding statistically significant data. In this manner, the multiparametric analysis of the physical,

biological, and chemical properties of each cell passing through the laser beam can be performed, yielding the information required to characterize the properties of the cells under study.

The Flow Cytometer

Figure 1 illustrates a generic flow cytometer that has the ability to use a single argon ion laser to distinguish the light scatter properties of cells and up to three different fluorochromes associated with each cell. More-sophisticated flow cytometers use two or more lasers to examine additional fluorochromes. Some flow cytometers are cell sorters that have the capacity to physically separate cells out of a population and collect that specific cell population. However, most of the experiments described in this chapter only require an instrument with a single argon ion laser that has the capacity to simultaneously analyze the light scatter properties of cells and two or three cell-associated fluorochromes.

Flow Cytometric Analysis of Virus-Infected Cells in Culture

Flow cytometric analysis of virus-infected tissue culture cells is becoming an extremely useful tool for studying virus-cell interactions, viral pathogenesis, and drug susceptibility testing (McSharry, 1994, 1995, 1998, 1999, 2000a, 2000b, 2000c). A typical flow cytometric analysis of uninfected and hCMV-infected human foreskin fibroblasts (HFF) is shown in Fig. 2. Monolayers of HFF grown in 25-cm^2 flasks were either mock infected or infected with hCMV. After 72 to 96 h of incubation at 37°C in 5% CO_2, the cells were removed from the monolayer with trypsin-EDTA, permeabilized with methanol, treated with fluorescein isothiocyanate (FITC)-labeled monoclonal antibody to the hCMV immediate-early (IE) antigens (MAB810; Chemicon International, Inc., Temecula, CA), and analyzed for the percentage of IE antigen-positive cells by flow cytometry. This monoclonal antibody recognizes an epitope that is shared by both hCMV IE-1 and IE-2 antigens and has been widely used in diagnostic virology for confirmation of hCMV infection in tissue culture (Mazeron et al., 1992). The left hand panels in Fig. 2 are dot blots of the flow cytometric analysis of the light scatter properties (FW-SC versus RT-SC) of uninfected (A) and hCMV-infected (B) cells. Flow cytometric analysis of the light scatter properties of the cells is used to separate intact cells from debris. The cells are collected, and a gate is placed around approximately 10,000 events that possess the light

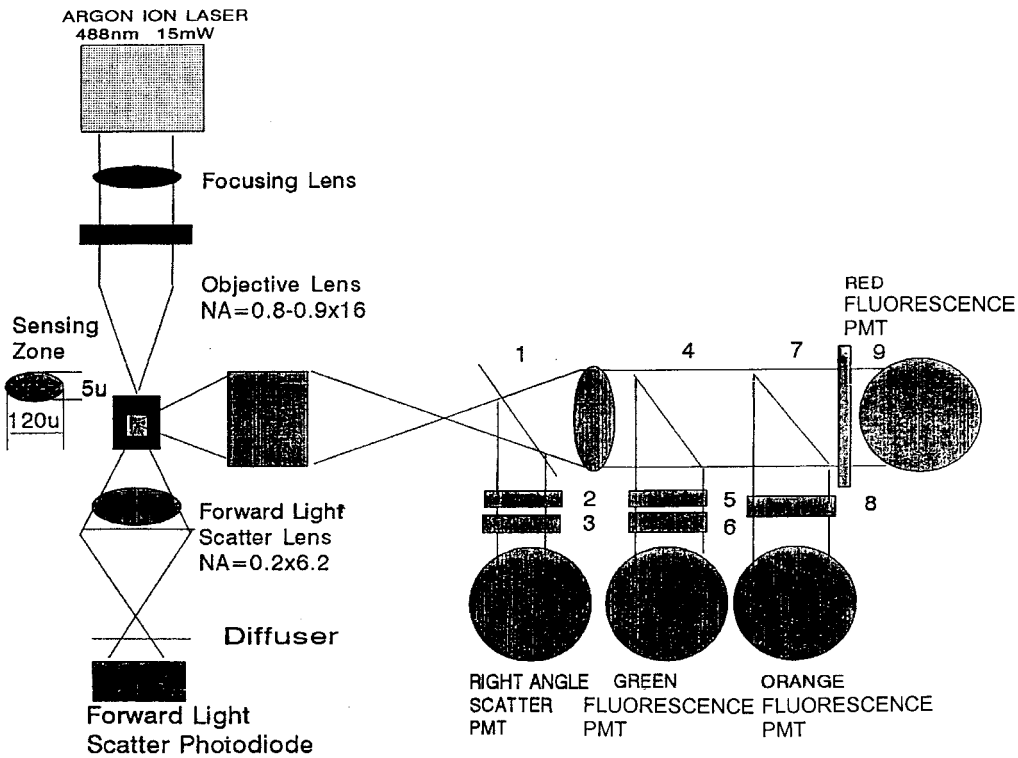

FIGURE 1 Diagram of a flow cell with attached optical systems. As the cells pass through the flow cell (the small black square box in the center left), a laser beam intersects the stream of cells and scatters light. FW-SC passes through the forward light scatter lens, and the energy is collected by the forward light scatter photodiode. RT-SC passes through the objective lens, the beam splitter, the laser line filter, and the diffuser, and the energy is collected by the right angle scatter photomultiplier tube (PMT). If the cells are labeled with fluorescent molecules, the laser will excite these molecules, which emit light of higher energies. The emitted energies of different wavelengths pass through the objective lens and various filters and are collected and amplified by the various PMTs. The amplified signals are converted into digital information and stored in a computer for further analysis. Numbers 1 through 9 refer to the following: 1, beam splitter; 2, laser line filter, 396- to 496-nm band pass; 3, diffuser; 4, dichroic mirror 1, 570-nm long pass; 5, laser cut filter, 490-nm short cut; 6, green filter, 515- to 530-nm band pass; 7, dichroic mirror 2, 610-nm long pass; 8, orange filter, 565- to 592-nm band pass; 9, red filter, 660-nm long pass. (From McSharry, 1994, with permission.)

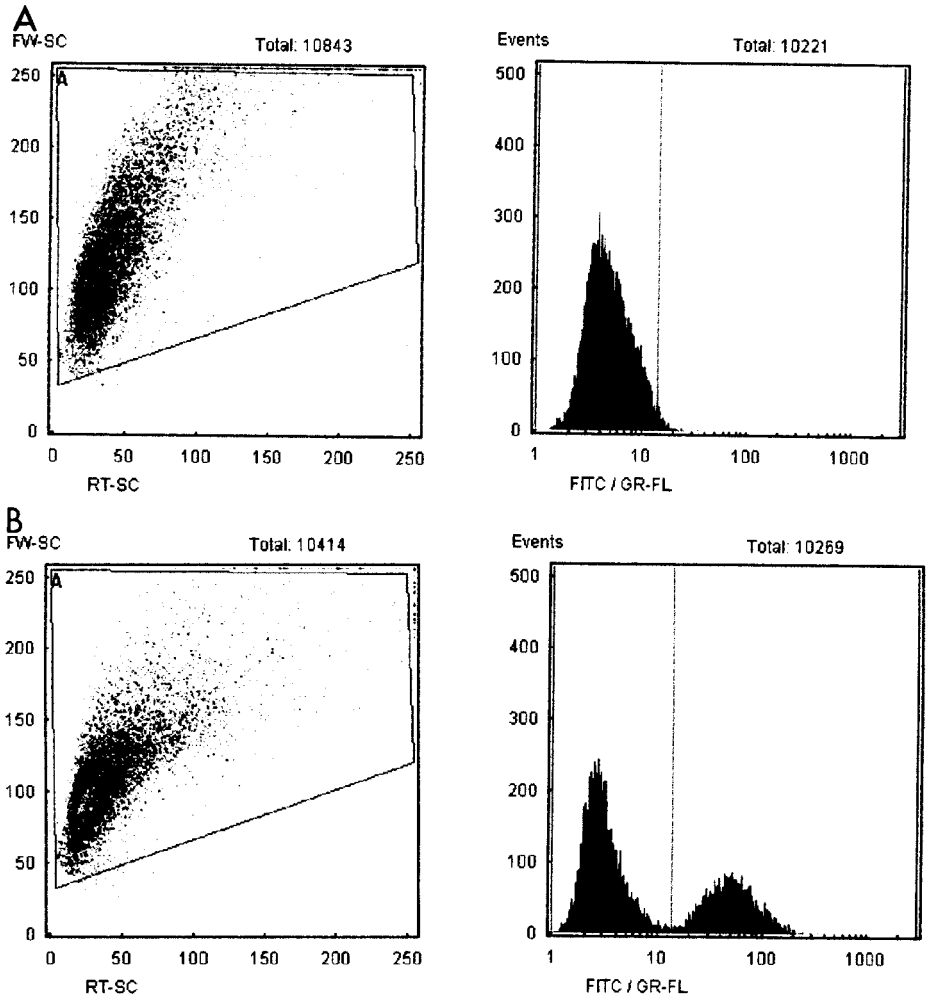

FIGURE 2 Flow cytometric analysis of uninfected and hCMV-infected HFF treated with FITC-labeled monoclonal antibody to the hCMV IE antigens. Uninfected (A) and hCMV-infected (B) HFF were permeabilized with methanol, treated with an FITC-labeled monoclonal antibody to the hCMV IE antigens, and analyzed for the percentage of antigen-positive cells by flow cytometry. The left-hand panels represent the forward and right angle light scatter analysis of the cells. Cells of the correct size to be intact cells are gated. The left-hand panels represent the analysis of fluorescence intensity of uninfected and hCMV-infected cells.

scatter properties characteristic of intact cells. The right-hand panels in Fig. 2 are histograms of the flow cytometric analysis of the fluorescence intensity associated with uninfected HFF (Fig. 2A) and hCMV-infected cells (Fig. 2B) within the gates illustrated in the respective left-hand panels. The histogram for uninfected cells (Fig. 2A) shows a homogenous population of cells with low FITC fluorescence intensity (FITC/GR-FL, between 1 and 10 on the *x* axis), indicating that uninfected cells do not contain the hCMV IE antigens. The histogram for hCMV-infected cells (Fig. 2B) shows two peaks, one with low fluorescence intensity (FITC/GR, between 1 and 10 on the *x* axis), similar to that for uninfected cells (Fig. 2A), and a second peak of higher fluorescence intensity (FITC/GR-FL, between 10 and 110 on the *x* axis), representing hCMV-infected cells that are synthesizing the IE antigens. These results show that the use of this monoclonal antibody to the hCMV IE antigens and flow cytometry can easily distinguish between the uninfected and hCMV-infected cell populations. Furthermore, the analysis will determine the percentage of cells in the uninfected and hCMV-infected cell populations, information that can be used to determine the extent of virus replication in this population of cells and, if the experiment is performed in the presence of antiviral drugs, the effect of drugs on virus replication. This procedure has been used by me and others to determine the susceptibilities of different viruses to antiviral compounds. Some of those experiments will be presented below. The flow cytometric analysis of virus-infected cells is rapid, quantitative, objective, and easily performed. The following sections will describe some of the current uses of flow cytometry in virology.

Since this field was last reviewed (McSharry, 2000a, 2000b, 2000c), flow cytometry has been used to study virus replication in rabies virus-infected cells (Bordignon et al., 2002), vaccinia virus-infected cells (Dominguez et al., 1998), dengue virus-infected cells (Ho et al., 2001; Kao et al., 2001;

Lambeth et al., 2005; Lee et al., 2006; Sanchez et al., 2006), influenza virus-infected cells (McSharry et al., 2004), and HIV-infected cells (McSharry et al., 2001a; Huerta et al., 2002). In addition, flow cytometry has been used to determine the effects of virus infection on cell cycle kinetics (Asmuth et al., 2005) and cellular DNA synthesis (Asmuth et al., 2005) and to identify cell surface receptors for viruses (Triantafilou et al., 2001; Freistadt and Eberle, 2006). Flow cytometry also has been used to detect baculovirus aggregates (Jorio et al., 2006). In this chapter, I will concentrate on a few studies that have used flow cytometry to measure in vitro drug susceptibility testing of antiviral compounds for cells infected with hCMV, human immunodeficiency virus (HIV), human herpesvirus 6 (HHV-6) and HHV8, Epstein-Barr virus (EBV), and influenza A and B viruses. Finally, I will mention some studies where flow cytometry has been used to study the innate and adaptive immune responses to virus infection (Kaur et al., 2007; Martinelli et al., 2007; Lee et al., 2007).

Apoptosis

Infection of cells with a number of viruses induces apoptosis (programmed cell death) (O'Brien, 1998; Everett and McFadden, 1999). In contrast, some viruses produce viral proteins in virus-infected cells that delay or inhibit apoptosis (Teodoro and Branton, 1997; Griffin and Hardwick, 1999). An early event in apoptosis is the redistribution of phosphoserine from the inner leaflet to the outer leaflet of the plasma membrane (Zhang et al., 1997). Late events in apoptosis result in nuclear condensation, fragmented DNA, and changes in cell size. Each of these characteristics of apoptotic cells can be detected and quantified by flow cytometry. Fragmented DNA in apoptotic cells binds less propidium iodide (PI) than chromosome length DNA, and flow cytometry can be used to measure this property of apoptotic cells. The flow cytometric analysis of the binding of PI to DNA to demonstrate the presence of a PI binding peak with less fluorescence intensity than the normal G_0/G_1 peak in cells is a simple method for detecting apoptosis in cells (McSharry, 1994). In another procedure, fluorochrome-labeled nucleotides can be added to the free ends of the fragmented DNA by terminal deoxynucleotidyl transferase, and the apoptotic cells containing fragmented DNA can be detected and quantified by flow cytometry (Bromidge et al., 1995; Chapman et al., 1995). Interactions between the Fas receptor and the Fas ligand lead to apoptosis in activated T cells (Kaplan and Sieg, 1998). The increased expression of the Fas receptor on the cell surface can be detected and quantified with fluorochrome-labeled monoclonal antibody to Fas antigen and flow cytometry. Finally, treatment of cells undergoing apoptosis with fluorochrome-labeled annexin V, a protein that binds to phosphoserine, followed by flow cytometry can detect and quantify the apoptotic cells with phosphoserine on the outside of the cell surface (Zhang et al., 1997). Flow cytometric analysis of these four properties of apoptotic cells has been used to detect and quantify the number of HIV-infected cells undergoing apoptosis. Furthermore, it is often possible to label cell surface markers with a fluorochrome-labeled monoclonal antibody so that the phenotype of the apoptotic cells can be determined or to use fluorochrome-labeled monoclonal antibodies to viral antigens to determine whether virus-infected cells undergo apoptosis or if uninfected bystander cells undergo apoptosis.

Cell Cycle Analysis

Normal resting cells have 2N DNA content before DNA synthesis and 4N DNA content after DNA synthesis. The binding of PI to nucleic acids (DNA and RNA) is directly proportional to the amount of nucleic acid present in the cell. If permeabilized cells are treated with RNase, then PI treatment can be used to measure the amount of DNA in a cell. Thus, the flow cytometric analysis of the fluorescence intensity of cells treated with RNase and PI can be used to determine their DNA content (McSharry, 1994). Flow cytometric analysis of normal resting cells treated with RNase and PI yields a histogram with a peak of low fluorescence intensity representing cells in the G_0/G_1 phase of the cell cycle, followed by a smaller peak with twice the fluorescence intensity of the first peak representing cells in the G_2/M phase of the cell cycle. The space between the two peaks represents cells in the S phase of the cell cycle. Flow cytometric analysis of PI binding to DNA in methanol-permeabilized, RNase-treated cells has been used to determine the ploidy of normal and cancer cells (Shapiro, 1995). It can also be used to determine the effect of virus infection on the cell cycle. Some examples of the use of flow cytometry to measure the effect of virus infection on the cell cycle are presented below.

Infection of a variety of human cells with HIV results in cell cycle arrest in the G_2 phase of the cell cycle. Analysis of mutant viruses suggested that arrest was associated with the Vpr accessory protein of HIV. Transfection of cells with plasmids containing the wild-type Vpr gene results in G_2 arrest followed by apoptosis (Stewart et al., 1997; Hrimech et al., 1999). Furthermore, virus particles containing Vpr were able to induce G_2 arrest in the presence of inhibitory concentrations of reverse transcriptase and protease inhibitors, suggesting that virus replication is not required to arrest cells in the G_2 phase of the cell cycle (Poon et al., 1998).

Human cytomegalovirus infection of fibroblasts arrests cells in the G_1 phase of the cell cycle (Bresnahan et al., 1996; Dittmer and Mocarski, 1997; Salvant et al., 1998). The UL69 gene product, a component of the virus particle, is at least partially responsible for arresting infected cells in at the G_1/S phase of the cell cycle (Lu and Shenk, 1999). Similar results have been reported for herpes simplex virus (de Bruyn Kops and Knipe, 1988) and Epstein-Barr virus (Cayrol and Flemington, 1996).

When a virus infects cells, the virus attempts to take over the cell to the virus's advantage. In the cases reported above, infection of cells with HIV blocked the cell cycle in G_2, a phase of the cell cycle where large amounts of transactivating factors required for HIV growth have accumulated in the cell. These factors enable HIV to replicate more efficiently (Goh et al., 1998). A similar situation exists in hCMV-infected cells that are arrested in the G_1 phase of the cell cycle. Since herpesviruses encode many of the genes required for viral DNA replication, all that is necessary is for the cell to contain large amounts of the building blocks for DNA synthesis. This is the case when cells are in G_1. Then all the virus has to do is to use the large pool of nucleotides for the synthesis of viral DNA.

These papers are excellent examples of the use of flow cytometry to study virus-cell interactions that interfere with the cell cycle and should give the reader interested in studying the effect of virus infection on cell cycle points of reference for their studies.

Drug Susceptibility Testing in Virus-Infected Cells

CMV-Infected Cells

Flow cytometry has been used to determine the drug susceptibilities of hCMV laboratory strains and clinical isolates to antiviral drugs (McSharry et al., 1998a; McSharry et al.,

1998b; McSharry, 2000a, 2000b, 2000c). Since hCMV clinical isolates are notoriously cell associated when grown in tissue culture cells, a drug susceptibility assay was developed for cell-associated hCMV. To perform the assay, medium containing various concentrations of drug is added to HFF monolayers in 25-cm^2 flasks. Then the monolayers are infected with hCMV-infected cells at a multiplicity of infection (MOI) of 0.001 to 0.01 virus-infected cell per uninfected cell by adding the infected cells directly to the medium. After incubation at 37°C for 144 h, the medium was removed, the cell monolayers from mock-infected or virus-infected flasks were removed with trypsin-EDTA, suspended with minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), centrifuged at 400 × *g* for 10 min, washed once with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}, and put on ice for 60 min. The cells were permeabilized by the addition of ice-cold absolute methanol to the residual PBS in the tube to yield a final concentration of 90% methanol. The permeabilized cells were stored at –70°C until they were prepared for analysis by flow cytometry.

To treat the permeabilized cells with monoclonal antibodies, the cells were centrifuged at low speed, the methanol was removed, and the cells were washed once with PBS without Ca^{2+} and Mg^{2+}. Monoclonal antibodies (FITC-labeled monoclonal antibody to the hCMV IE antigens, MAB810, or FITC-labeled monoclonal antibody to the hCMV gB late antigen, MAB8126, obtained from Chemicon International, Inc., Temecula, CA) diluted to the appropriate concentration in 0.007% Evans blue containing 0.1% bovine serum albumin (BSA) and sodium azide (Chemicon International, Inc.) were added to the permeabilized cells, and the cells were incubated for 60 min at 37°C. After the incubation period, the cells were washed with 3× PBS-Tween 20 (Chemicon International, Inc.) and analyzed by flow cytometry.

An Ortho Cytoron absolute analytical flow cytometer (Ortho Diagnostic Systems, Inc., Raritan, NJ), with a 15-mW argon ion laser set at 488 nm for excitation, was used for these studies. The initial analysis was FW-SC versus RT-SC to distinguish intact cells from smaller debris. A gate was drawn around the events representing intact cells, and the events within the gate were analyzed for FITC-labeled fluorescence intensity. The results are presented as the percent of antigen-positive cells in the analyzed population. Controls consisted of uninfected cells treated with the FITC-labeled monoclonal antibodies to IE or late antigens and hCMV-infected cells treated with FITC-labeled isotype control antibodies. Figures 3 and 4 illustrate the analysis of the effect of foscarnet on the percentage of cells expressing the IE (Fig. 3) or late (Fig. 4) antigens. In the absence of foscarnet, 34% of the cells synthesized the IE antigen, and in the presence of 200 μM foscarnet, only 17% of the cells synthesized the IE antigen. In the absence of foscarnet, 24.9% of the cells synthesized the late antigen, and in the presence of 200 μM foscarnet, only 11.7% of the cells synthesized the late antigen. On the basis of this type of analysis for each of six drug concentrations, the 50% effective concentration (EC_{50}) values for this hCMV clinical isolate were calculated to be 99.58 μM foscarnet based the analysis of the percentage of cells synthesizing the IE antigen and 102.06 μM foscarnet based on the percentage of cells synthesizing the late antigen. The two EC_{50}s are in excellent agreement and demonstrate that, under conditions of low MOI for hCMV-infected cells, EC_{50}s can be determined using either the effect of drugs on the synthesis of the IE antigen or the late antigen. Under conditions of high MOI, analysis of the effect of drugs on IE antigen synthesis can be performed on virus-infected cells after only 24 h of incubation, making it a more rapid assay than the plaque reduction assay (PRA), which takes up to 1 week for countable plaques to form on the infected monolayer. The EC_{50} for this clinical isolate by the PRA was 91.27 μM foscarnet. These results show that under conditions of low MOI (0.01) the EC_{50}s derived from the flow cytometry drug susceptibility assay using monoclonal antibodies to either the IE or late antigens are in excellent agreement with the EC_{50}s obtained with the PRA.

One drawback of the flow cytometry drug susceptibility assay is that it does not always yield an EC_{90} or EC_{95} value because of the presence of input virus-infected cells. Despite this drawback, the speed, accuracy and ease of the assay far outweigh any of its disadvantages.

Figure 5 illustrates the flow cytometric analysis of the effect of foscarnet on the percentage of cells synthesizing the IE antigen in cells infected with foscarnet-susceptible and foscarnet-resistant hCMV clinical isolates in the presence of 0 or 800 μM drug. The upper panels show the effect of foscarnet on a drug-sensitive clinical isolate, and the lower panels show the effect of foscarnet on a drug-resistant clinical isolate. The analysis shows that, in presence of 0 μM foscarnet, 28.9% of the cells infected with a foscarnet-sensitive clinical isolate synthesized the IE antigen and, in the presence of 800 μM foscarnet, only 7.2% of the cells synthesized the IE antigen. When cells infected with a foscarnet-resistant clinical isolate were analyzed (lower panels), 95.0% of the cells were IE antigen positive in the absence of foscarnet and 72.3% of the cells were antigen positive in the presence of 800 μM foscarnet. For the foscarnet-resistant clinical isolate, there was less than a 50% reduction in the percentage of antigen-positive cells, showing that this isolate is more resistant to foscarnet than the drug-susceptible clinical isolate used in this experiment. These results show that the assay can clearly distinguish between foscarnet-sensitive and -resistant clinical isolates. There is excellent correlation between the EC_{50}s for foscarnet for drug-susceptible or drug-resistant isolates obtained using the flow cytometry assay and the PRA.

In summary, the flow cytometry drug susceptibility assay for hCMV clinical isolates is accurate, rapid, and quantitative and can be automated. It can be used to measure the susceptibility of hCMV clinical isolates to antiviral compounds. The assay readily distinguished between drug-susceptible and drug-resistant clinical isolates. Finally, the assay yields EC_{50}s that are equivalent to those obtained with the PRA.

This technique has been used to show the susceptibilities of ganciclovir-susceptible and ganciclovir-resistant hCMV laboratory strains and clinical isolates to the L-riboside, 1263W94 (McSharry et al., 2001a), and the nonnucleoside inhibitors, BAY38-4766 and BAY43-9695 (McSharry et al., 2001b). Comparison of the EC_{50}s of these compounds for hCMV derived from the flow cytometry drug susceptibility assay and the standard PRA showed excellent correlation between the two methods. The distinct advantage of the flow cytometry drug susceptibility assay over the PRA was speed and accuracy. Using a fluorochrome-labeled monoclonal antibody directed against the hCMV IE antigen, virus-infected cells could be detected and quantified within 24 h of infection by flow cytometry. FACS analysis is a rapid, effortless method for quantifying the number of virus-infected cells and the effect of antiviral compounds on the number of virus-infected cells. In contrast, determination of the effect of antiviral compounds on hCMV replication by the PRA took 4 to 6 days for plaques to appear on the monolayer, and even after fixing and staining the infected cell monolayer with crystal violet in ethanol to visualize the plaques, counting

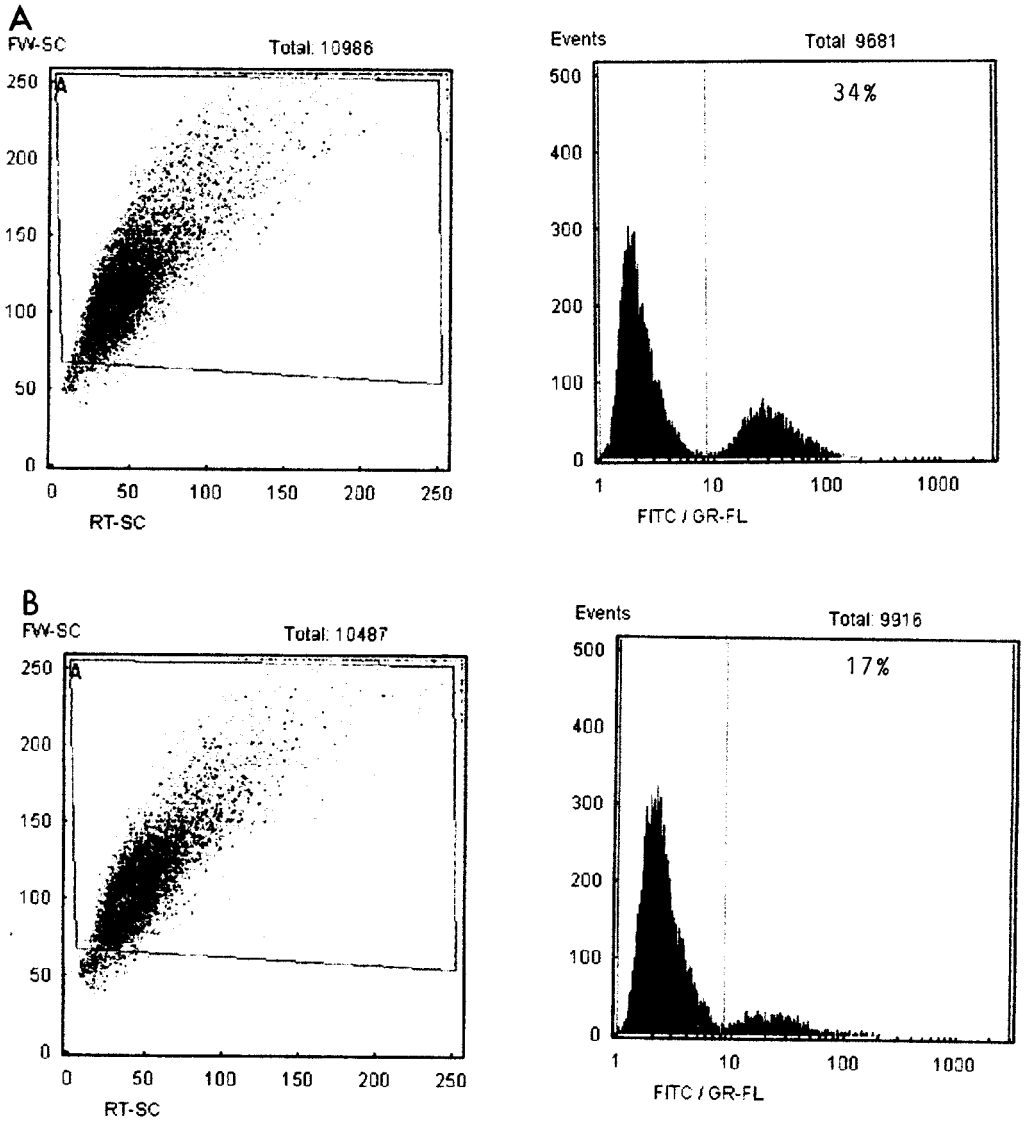

FIGURE 3 Effect of foscarnet on hCMV IE antigen synthesis in cells infected with a foscarnet-susceptible hCMV clinical sample. Medium containing 0 (A) or 200 (B) μM foscarnet was added to HFF monolayers. Virus-infected cells were added to the flask at an MOI of 0.01. After incubation at 37°C for 144 h, the cells were harvested with trypsin-EDTA, permeabilized with methanol, treated with a monoclonal antibody to the hCMV IE antigens, and analyzed by flow cytometry for the percentage of antigen-positive cells. Initially, the cells were analyzed for forward angle light scatter (FW-SC) versus right angle light scatter (RT-SC) to identify intact cells and exclude debris. The intact cells were gated and analyzed for the percentage of antigen-positive cells in the population. In the absence of foscarnet, 34% of the cells synthesized the hCMV IE antigens. In the presence of 200 μM foscarnet, only 17% of the cells expressed the hCMV IE antigens.

the plaques with the aid of a light microscope is very labor intensive and leads to inaccurate plaque counts. The key element in this FACS assay is the availability of commercially available fluorochrome-labeled monoclonal antibodies to hCMV IE and late antigens that clearly distinguish between virus-infected and uninfected cells. The ability to count 10,000 events within less than a minute makes the flow cytometry-based drug susceptibility assay a very useful and accurate tool in the area of drug discovery.

Flavivirus-Infected Cells

A flow cytometry-based drug susceptibility assay was developed for heparin sulfate mimetics for dengue virus, West Nile virus, Murray Valley encephalitis virus, and Japanese encephalitis virus (Lee et al., 2006). BHK cell monolayers in six-well tissue culture plates were preincubated with Hanks' balanced salt solution with BSA (HBSS-BSA) containing various concentrations of inhibitors for 15 min at 37°C, 5% CO_2. The viruses, diluted to yield an MOI of 0.5 PFU per cell,

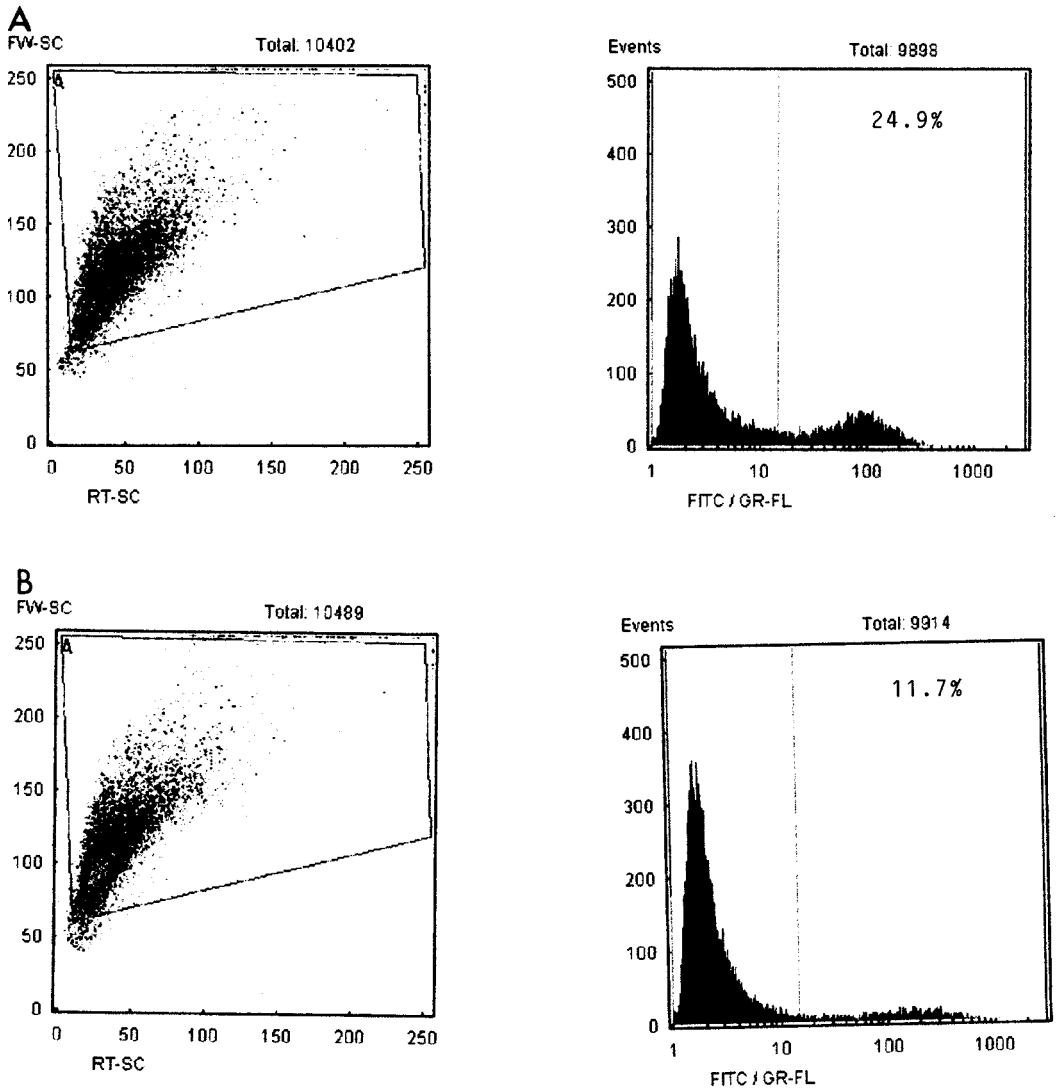

FIGURE 4 Effect of foscarnet on hCMV late antigen synthesis in cells infected with a foscarnet-susceptible hCMV clinical isolate. Same as in Fig. 3, except that a monoclonal antibody to the hCMV late antigen was used to identify the antigen-positive cells. (A) In the absence of foscarnet, 24.9% of the cells were positive for the late antigen. (B) In the presence of 200 μM foscarnet, only 11.7% of the cells were positive for the late antigen.

were also preincubated for 15 min at room temperature with the same concentrations of inhibitors prior to addition of the virus to the cell monolayers. The preincubated viruses were added to the cell monolayers and adsorbed at 37°C, 5% CO_2 for 1 h. Then the input virus was removed, the monolayers were washed, virus growth medium was added to the infected monolayers, and the cells were incubated at 37°C, 5% CO_2 for 16 to 18 h. The cells were harvested with trypsin-EDTA solution, collected by centrifugation, and fixed and permeabilized with ice-cold 75% ethanol for 30 min at 4°C. Then the cells were suspended in MEM–5% FBS containing antibodies specific for the E or NS1 proteins for 1 h at 4°C, washed, and then treated with FITC-conjugated sheep anti-mouse immunoglobulin G (IgG). The cells were washed to remove excess antibody and analyzed for the percentage of antigen-positive cells by FACS analysis. Uninfected cells served as controls. The results of this study showed that the heparin sulfate mimetics inhibited flavivirus replication in vitro.

Lymphotropic Herpesvirus-Infected Cells

The lymphotropic herpesviruses, EBV, HHV-6, and HHV-8, cause lymphoproliferative disorders and cancers. There has been an extensive search for antiviral compounds that would prove effective in the treatment of these diseases. Traditional assays for measuring the effect of antiviral compounds for this group of viruses have included indirect immunofluorescence assays (IFA), enzyme-linked immunosorbent assays (ELISA), and in situ DNA hybridization assays. These assays are time-consuming and labor-intensive or not very sensitive. In an attempt to develop faster, more quantitative, and less labor-intensive assays, Long et al. (2003) developed flow cytometry-based assays for studying the effects of antiviral compounds for each of these lymphotropic herpesviruses.

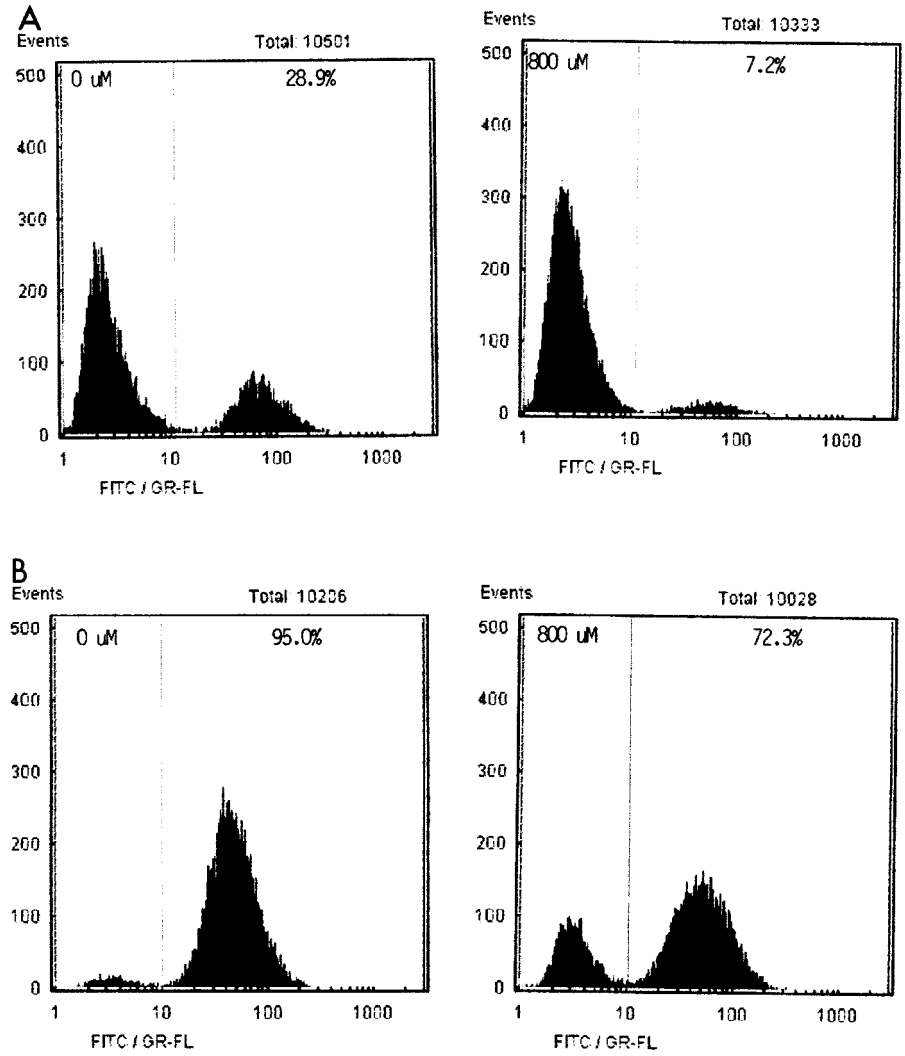

FIGURE 5 Effect of foscarnet on the synthesis of IE antigen in cells infected with a foscarnet-susceptible hCMV clinical isolate (A) or a foscarnet-resistant hCMV clinical isolate (B). Infection was performed as described in the legend to Fig. 3. Of the cells infected with the foscarnet-susceptible clinical isolate, 28.9% synthesize the IE antigen in the absence of foscarnet, whereas only 7.2% of the cells synthesize the IE antigen in the presence of 800 μM foscarnet. Of the cells infected with the foscarnet-resistant clinical isolate, 95.0% synthesize the IE antigen in the absence of foscarnet, whereas 72.3% of the cells infected with the resistant isolate synthesize the IE antigen in the presence of 800 μM foscarnet.

The effect of antiviral compounds on the replication of EBV was studied in Daudi cells, a Burkitt's lymphoma-derived B cell line latently infected with EBV, and H1 cells, a subclone of human P3HR-1 cells that continuously produces EBV. Daudi cells were superinfected with a sufficient amount of EBV to infect 10% of the cells, and 100% of the H1 cells were EBV infected. The infected cells were treated with various concentrations of cidofovir, ganciclovir, acyclovir, foscarnet, or pencyclovir. At the appropriate times, cells were collected, fixed and permeabilized with ice-cold methanol, and treated with a primary antibody to the EBV capsid antigen, glycoprotein 125, followed by an FITC-labeled goat anti-mouse IgG and IgM antibody. Then the cells were analyzed by flow cytometry to determine the number of EBV-infected cells. The results showed that acyclovir inhibited EBV replication in Daudi and H1 cells, whereas cidofovir, ganciclovir, foscarnet, and pencyclovir did not inhibit virus replication. Analysis of the effect of these compounds on EBV replication using the more standard techniques of DNA hybridization, ELISA, or IFA yielded the same overall result. For example, the EC_{50}s for acyclovir for EBV obtained by FACS assay and IFA were identical (1.3 μg/ml). Using a similar approach and different antibodies, the effects of these antiviral compounds on the replication of HHV-6A in HSB-2 cells, HHV-6B in Sup-T1 cells, and HHV-8 in BCBL-1 were examined by FACS analysis of virus-infected cells and by IFA. The results from both assays showed that cidofovir, ganciclovir, and foscarnet inhibited HHB-6A and HHV-6B replication, with both assays yielding similar EC_{50}s for each of these compounds. The effects of these compounds on the replication

of HHV-8 in a lymphoma-derived BCBL-1 cell line, induced into lytic HHV-8 expression by treatment with phorbol 12-myristate 13-acetate, were determined by FACS and IFA. The results showed that only cidofovir was effective in inhibiting HHV-8 replication, with the FACS assay and the IFA giving identical EC_{50}s. This paper clearly shows that the effect of antiviral compounds on lymphotropic herpesvirus replication in cells can be determined by flow cytometric analysis of virus-infected cells and that the FACS analysis gives EC_{50}s almost identical to the more labor-intensive IFA. These results show that the rapid, sensitive, quantitative FACS assay can be used to determine the drug susceptibilities of antiviral compounds for the lymphotropic herpesviruses. Since these viruses circulate in the peripheral blood of infected individuals, it may be possible to use flow cytometry to detect lymphocytes infected with these viruses in vivo and to determine the effect of these treatments on viral burden.

Influenza A and B Virus-Infected Cells

A flow cytometry-based drug susceptibility assay was developed for influenza A and B viruses for the neuraminidase inhibitors peramivir, oseltamivir carboxylate, and zanamivir and the nucleoside analogue ribavirin (McSharry et al., 2004). Various strains of wild-type and neuraminidase inhibitor-resistant influenza A viruses or influenza B viruses were diluted in MEM supplemented with 0.2% BSA, 2 μg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin and antibiotics (virus growth medium) to yield a virus concentration of 1,000 PFU/ml. Confluent MDBK cell monolayers were washed twice with virus growth medium, and the diluted virus was added to the monolayers to yield an MOI of 0.001 PFU per cell. After a 2-h incubation period, the inoculum was removed and 5 ml of virus growth medium containing various concentrations of neuraminidase inhibitor or ribavirin was added to each flask of virus-infected cells. The flasks were incubated at 35°C, 5% CO_2 until cytopathic effects (clusters of rounded cells) appeared on the monolayers infected in the absence of drug. At this time, the medium was removed and tested for the amount of virus released into the medium by plaque assay. In addition, the virus-infected cells were removed from the flask by treatment with trypsin-EDTA, the released cells were suspended in MEM containing FBS, collected by centrifugation, fixed and permeabilized with methanol, and treated with a fluorochrome-labeled monoclonal antibody to the influenza A or B virus nucleocapsid antigen. After washing away any unbound antibody, the stained cells were analyzed for the presence of influenza A or influenza B nucleocapsid antigens by FACS analysis. The results showed that, in the absence of antiviral compounds, virus replicated to a high titer, infecting greater than 90% of the cells over a 48-h period. With increasing concentrations of neuraminidase inhibitors or ribavirin, the virus yield, as determined by plaque assay, declined and the number of antigen-positive cells, as determined by FACS analysis, also declined. By plotting the decrease in the number of PFU or the percentage of antigen-positive cells versus the drug concentrations, EC_{50}s of each of the neuraminidase inhibitors and of ribavirin were determined. Both assays clearly distinguished between drug-susceptible and drug-resistant influenza virus strains. The advantage of the FACS drug susceptibility assay compared to the virus yield assay (plaque assay) is speed and ease of quantifying the results of the assay.

One major disadvantage of using the FACS assay for measuring the effect of neuraminidase inhibitors on influenza virus replication in cells is the ability of influenza viruses to spread from cell to cell even in the presence of effective concentrations of the neuraminidase inhibitors. Neuraminidase inhibitors block the release of virus from infected cells. This results in an accumulation of nucleocapsids in the cell and many hemagglutinin molecules in the plasma membrane. The hemagglutinin molecules on the outside of the plasma membrane allow virus-infected cells to fuse with neighboring cells in the monolayer (fusion from without). To avoid this problem with neuraminidase inhibitors, one must use a very low MOI (0.001 PFU/cell) and harvest the virus-infected cells before the entire monolayer becomes infected, usually within 24 to 36 h of infection. When the FACS drug susceptibility assay is used for compounds that prevent the synthesis of viral antigens such as ribavirin or adamantane derivatives, this is not a problem, since these inhibitors block virus replication at an early event, either at the uncoating step or at the RNA replication step, so no virus particles containing proteins with fusing activity accumulate in the cell. In summary, the FACS drug susceptibility assay for determining the effect of drugs on influenza virus replication is very good if the drug blocks virus replication by preventing synthesis of the viral antigens. It must be used with caution if the antiviral compound allows viral protein synthesis to occur and virus particles to accumulate in the cell. This caution may apply to other viruses that produce proteins which allow virus-infected cells to fuse with one another.

HIV-Infected Cells

A number of laboratories have developed methods for determining the susceptibilities of HIV laboratory strains and clinical isolates to antiretroviral drugs. These assays can be either genotypic assays that identify specific mutations associated with drug resistance (Shafer, 2002) or phenotypic assays that determine susceptibility of HIV to an antiviral compound irrespective of the genetic defect (Hirsch et al., 2000). The original phenotypic assays utilized activated peripheral blood mononuclear cells (PBMCs) obtained from HIV seronegative donors as targets for virus infection and required days to weeks for production of infectious virus that was detected by assaying for the presence of the HIV p24 antigen by ELISA (Japour et al., 1993). These phenotypic assays were time-consuming, labor-intensive, and expensive to perform. To speed up the process, homologous recombination phenotypic assays were developed that are more rapid (Hertogs et al., 1998). These assays require reverse transcription-PCR amplification of the genes of interest, and they tend to measure the predominant species present in the population. If the mutation is present in fewer than 25% of the transcripts, it may not be detected even with this very sophisticated assay.

Over the last several years, several phenotypic assays have been developed that rapidly determine drug susceptibilities of cell-free HIV clinical isolates. These assays measure the expression of a reporter gene such as luciferase (Petropoulos et al., 2000; Spenlehauer et al., 2001), green fluorescent protein (GFP) (Dorsky and Harrington, 1999; Gervaix et al., 1997; Lindsten et al., 2001; Zhang et al., 2004), or secretory alkaline phosphatase (Miyake et al., 2003) using a spectrophotometer or a flow cytometer, while others count the number of β-galactosidase-expressing blue cells (Pirounaki et al., 2000; Hachiya et al., 2001) in an HIV-infected monolayer of susceptible cells with the aid of a light microscope (Princen et al., 2004). We have developed a phenotypic flow cytometry-based drug susceptibility assay that can be used for X4 and R5 tropic HIV laboratory strains and clinical isolates. This phenotypic assay can be used for cell-free HIV

laboratory strains and clinical isolates or for cell-associated laboratory strains or clinical isolates of HIV (McSharry et al., 2001c). This assay uses osteosarcoma cells (R3/X4/R5) that express the CD4 receptor and both the X4 and R5 coreceptors for HIV on their cell surfaces. In addition, these cells have the HIV type 2 (HIV-2) long terminal repeat fused to the gene for GFP integrated into their genome. When HIV replicates in these cells, the Tat protein binds to the long terminal repeat, activating the expression of GFP. Thus, GFP-expressing cells represent HIV-infected cells. These GFP-expressing cells can be quantified by flow cytometry. We have used this phenotypic assay to determine the susceptibilities of cell-free or cell-associated HIV laboratory strains and clinical isolates to several different classes of antiretroviral compounds (McSharry et al., 2001c).

To determine if infection of R3/X4/R5 cell monolayers with HIV results in the expression of GFP detectable by FACS analysis, R3/X4/R5 cell monolayers were mock infected or infected with cell-free HIV_{IIIB} (150 ng/ml of p24 antigen). After 2 days of incubation, the cells were harvested and analyzed for the expression of GFP by FACS analysis (Fig. 6). Initially, the cells were analyzed for FW-SC versus RT-SC to distinguish between intact cells and debris. A gate was set to include the events representing intact cells and excluding debris. Then 10,000 events within the gate were analyzed for fluorescence intensity (*y* axis) versus FW SC (*x* axis).

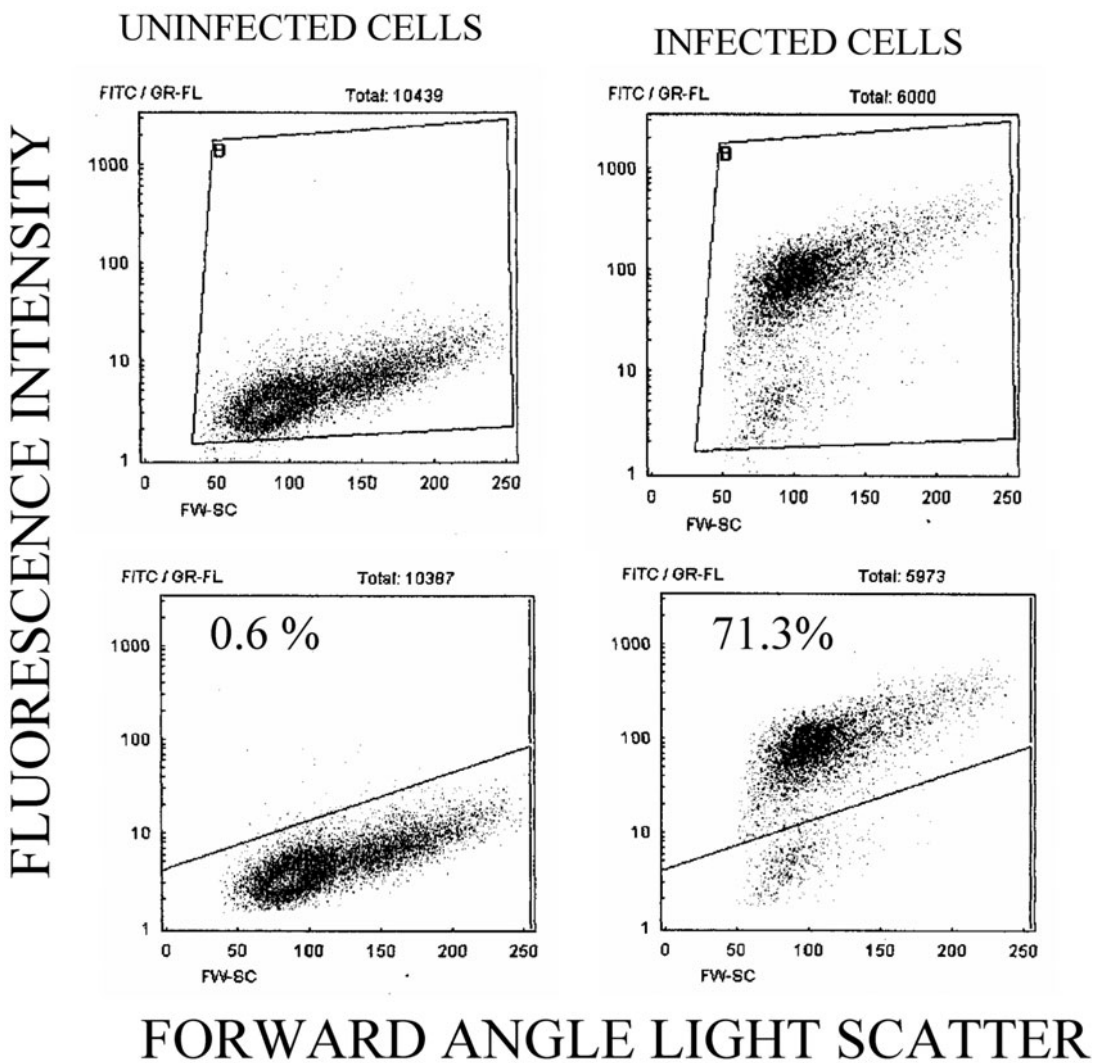

FIGURE 6 FACS analysis of uninfected and HIV-infected R3/X4/R5 cells expressing GFP. The left-hand side of the figure represents FACS analysis of uninfected R3/X4/R5 cell monolayers, and the right-hand side of the figure represents FACS analysis of R3/X4/R5 cell monolayers infected with cell-free HIV_{IIIB}. After 48 h of incubation at 37°C under an atmosphere of 5% CO_2, the cells were removed with trypsin-EDTA, fixed in 1% paraformaldehyde, and examined for GFP by FACS. Events with light scatter properties characteristic of intact cells were gated and analyzed for fluorescence intensity (FITC-GR-FL) versus forward angle light scatter (FW-SC). A gate was set on the uninfected cell population such that less than 1% of the fluorescent events were above the gate. Using this gate, 71.3% of the events were above the gate in the HIV_{IIIB}-infected cells.

Uninfected R3/X4/R5 cells were used to set a gate to distinguish the background fluorescence intensity associated with uninfected cells from that associated with HIV-infected cells. FACS analysis of uninfected R3/X4/R5 cells shows one population with low fluorescence intensity (Fig. 6, upper left-hand panel), whereas FACS analysis of HIV_{IIIB}-infected cells shows two populations, a small population with low fluorescence intensity and a large population with high fluorescence intensity (Fig. 6, upper right-hand panel). A gate was drawn above the events with the low fluorescence intensity so that less than 1% of the events in the uninfected cell population are above the gate (Fig. 6, lower left-hand panel). When this gate was applied to the HIV_{IIIB}-infected cell population, 71.3% of the events are found above the gate (Fig. 6, lower right-hand panel). Since only HIV-infected cells express high levels of GFP, these results indicate that 71.3% of the cells are infected by HIV_{IIIB}. To confirm that these GFP-expressing cells are actually infected with HIV, the media from the wells with uninfected or HIV-infected R3/X4/R5 cells were tested for the presence of HIV p24 antigen associated with released virus by ELISA. The medium from the uninfected cells had less than 1 ng/ml of p24 antigen (background), whereas the medium from the HIV_{IIIB}-infected cells had 110.8 ng/ml of p24 antigen. The GFP-FACS assay is rapid and does not require expensive fluorochrome-labeled monoclonal antibodies to detect the virus-infected cells. These results confirm previous demonstrations that HIV-infected R3/X4/R5 cells express GFP and that the percentage of GFP-expressing cells can be determined by FACS analysis (Gervaix et al., 1997).

To determine if this assay can be used to measure the drug susceptibilities of HIV for antiretroviral compounds, R3/X4/R5 cell monolayers were infected with a cell-free suspension of an azidothymidine (AZT)-susceptible HIV clinical isolate containing 200 ng of p24 antigen/ml in the absence and presence of various concentrations of AZT. The same gates shown in Fig. 6 were used to analyze the percentage of cells expressing GFP at each drug concentration. In the absence of AZT, 26.8% of the cells expressed GFP; in the presence of 0.025 μM AZT, 13.8% of the cells expressed GFP; in the presence of 0.05 μM AZT, 11% of the cells expressed GFP; and in the presence of 0.1 μM AZT, only 4.9% of the cells expressed GFP. By plotting the percentage of GFP-positive cells against the drug concentration using the Sigmoid Emax model (D'Argenio and Schumitzky, 1997), the EC_{50} of AZT for this clinical isolate was determined to be 0.034 μM. This value is consistent with EC_{50}s of AZT for drug-susceptible HIV clinical isolates determined by more time-consuming and costly phenotypic drug susceptibility assays (Japour et al., 1993).

To determine if cell-associated HIV can be used in this drug susceptibility assay, R3/X4/R5 cell monolayers were infected with H9 cells chronically infected with HIV_{IIIB} in the absence and presence of AZT. After 48 h of incubation, the cells were removed with trypsin-EDTA and analyzed for fluorescence intensity of cells expressing GFP versus FW SC by FACS analysis. The results are presented in Fig. 7. The left-hand panels represent FACS analyses of R3/X4/R5 cells infected in the absence of AZT, and the right-hand panels represent FACS analyses of R3/X4/R5 cells infected in the presence of 0.05 μM AZT. The upper panels show three populations of cells: one with essentially no GFP fluorescence intensity, a population with low GFP fluorescence intensity, and a population with high GFP fluorescence intensity. A gate was set to exclude the HIV_{IIIB}-infected H9 cells that exhibit essentially no fluorescence intensity from the analysis while including both the uninfected R3/X4/R5 cells (low-fluorescence-intensity cells) and the HIV-infected R3/X4/R5 cells (high-fluorescence-intensity cells). To determine the percentage of R3/X4/R5 cells expressing high fluorescence intensity, a gate was set between the populations of R3/X4/R5 cells with low fluorescence intensity and those with high fluorescence intensity (Fig. 7, lower panels). Using this gate, the data show that, in the absence of AZT, 71.4% of the cells express GFP, whereas in the presence of 0.05 μM AZT, only 35.1% of the cells express GFP. These results show that this assay can be used to determine the drug susceptibility of cell-associated HIV to AZT. Activation of GFP in the R3/X4/R5 cells was not due to cell-free virus derived from the H9 cells chronically infected with HIV_{IIIB} because the chronically infected cells do not release detectable amounts of HIV, as determined by p24 antigen ELISA. Furthermore, any cell-free HIV_{IIIB} was removed from the culture medium by centrifugation before the chronically infected cells were added to the R3/X4/R5 cell monolayers. Thus, direct spread of HIV from the chronically infected $H9_{IIIB}$ cells to the R3/X4/R5 cells resulted in infection of the R3/X4/R5 cells and the expression of GFP in these target cells. We have not demonstrated that this FACS assay could be used to detect HIV-infected cells in peripheral blood or lymphoid organs of HIV-infected individuals, but this in vitro demonstration suggests that it may be possible to detect HIV-infected cells in AIDS patients and to determine the drug susceptibilities of HIV in the virus-infected cells obtained from these individuals. The presence of archival viruses will have to be addressed, but this assay may lead to a procedure to address this issue.

Miscellaneous Flow Cytometry Assays for Viruses

In addition to the use of flow cytometry for drug susceptibility testing, flow cytometry has been used to detect virus-infected cells for other purposes. Several examples are cited.

Dengue Virus

FACS analysis has been used to detect dengue virus in clinical samples and virus-infected cell lines (Ho et al., 2001; Kao et al., 2001; Marovich et al., 2001). Lambeth et al. (2005) showed that flow cytometric analysis of dengue virus-infected C6/36 *Aedes albopictus* mosquito cells could be used to monitor virus infection. For this study, various dilutions of dengue virus 2 or dengue virus 3 were diluted in virus growth medium and inoculated onto C6/36 mosquito cells. After incubation at 28°C for various times, the cells were harvested, fixed, permeabilized, and treated with an FITC-labeled monoclonal antibody to a flavivirus group-specific antigen. The treated cells were analyzed for the number of antigen-positive cells by flow cytometry. The data show that for dengue 2 virus-infected cells, the percentage of antigen-positive cells rose from 7.54% at day 1 to 52.77% at day 7. Similar results were shown for the dengue 3 virus-infected cells. Uninfected cells showed no antigen-positive cells, indicating the specificity of the monoclonal antibody. When the infection was initiated at high MOI and terminated at 24 h postinfection to avoid second rounds of virus replication, the assay could be used to titer a stock of virus. The FACS assay for determining virus titer was more efficient than the standard plaque assay in that it is faster (1 day versus 7 days) and easier to quantitate. Furthermore, essentially all strains of dengue virus tested could grow in this mosquito cell line.

These authors went on to use this assay to measure antibody neutralization of dengue virus. They compared the

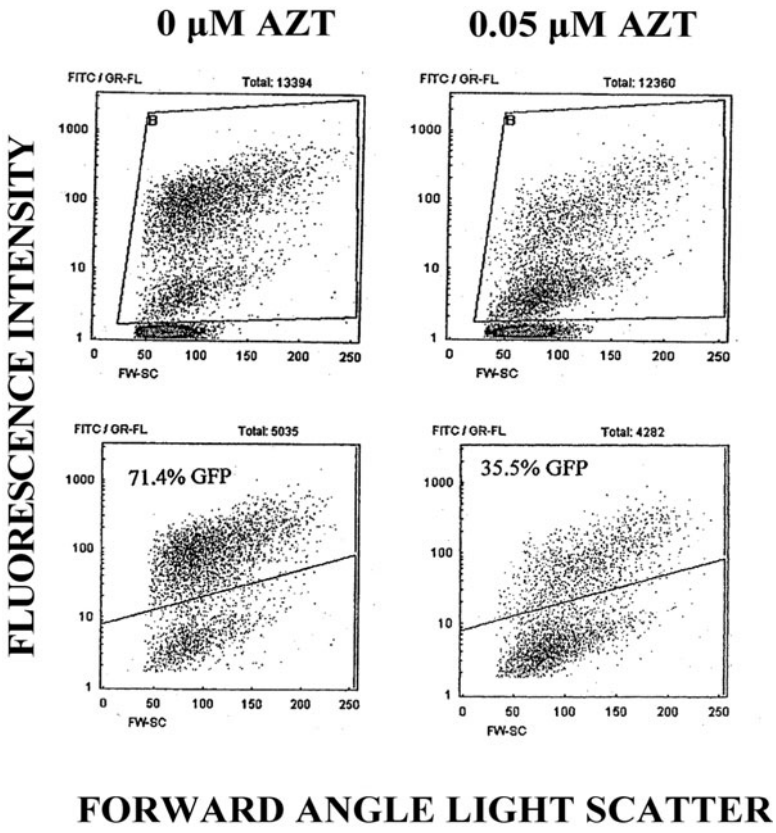

FIGURE 7 Cell-associated HIV drug susceptibility assay. R3/X4/R5 cell monolayers were infected with H9 cells chronically infected with HIV_{IIIB} in the absence (left-hand panels) and presence (right-hand panels) of 0.05 μM AZT. After two days of incubation, the cells were analyzed for the percentage of cells expressing high levels of fluorescence intensity by FACS. The HIV_{IIIB}-infected cells, which do not express GFP, were gated out and the R3/X4/R5 cells expressing low and high levels of fluorescence intensity were included in the gate for analysis of fluorescence intensity. To determine the percentage of HIV-infected R3/X4/R5 cells, a gate was drawn between the cell population with low fluorescence intensity and the cell population with high fluorescence intensity. In the absence of AZT, 71.4% of the R3/X4/R5 cells expressed GFP, whereas in the presence of 0.05 μM AZT, only 35.5% of the R3/X4/R5 cells expressed GFP.

FACS assay with the standard plaque reduction test in Vero cells. Antibody was diluted and incubated with dengue 2 virus. After incubation, the virus present in each antibody dilution was assayed for the number of infectious viruses by the FACS assay in 24 h and the plaque neutralization assay in 7 days. Both assays gave similar neutralization titers, but the FACS assay gave an answer in 1 day, whereas the plaque neutralization assay took 7 days. This is another example of the benefits of FACS assays over the more standard assays. A similar type of neutralizing antibody assay for dengue virus was developed by Martin et al. (2006).

HIV

Several laboratories have demonstrated that flow cytometric analysis of HIV-infected PBMCs treated with fluorochrome-labeled monoclonal antibodies directed against the Gag antigen can be used to detect and quantify HIV-infected PBMCs in vitro and in vivo (McSharry et al., 1990; Ohlsson-Wilhelm et al., 1990). More recently, flow cytometric analysis of HIV-infected PBMCs has been used to determine the neutralization titer of plasma obtained from HIV-seronegative and HIV-seropositive people (Darden et al., 2000). In this virus neutralization study, diluted normal human serum or sera obtained from people infected with subtype B or E HIV were added to wells of a 24-well plate. Subtype B or E virus was added to the wells and incubated for 30 min. Then phytohemagglutinin-activated, CD8-depleted PBMCs were added to the wells, and the plates were incubated at 37° for 4 days. At that time, the cells were fixed, permeabilized, stained with an FITC-labeled monoclonal antibody to the HIV p24 antigen, and assayed for the presence of p24 antigen-positive cells by FACS analysis. The results showed that sera from individuals infected with subtype B virus neutralized subtype B virus but not subtype E virus, and sera from patients infected with subtype E virus neutralized subtype E virus but not subtype B virus. This assay took only 4 days to complete. The standard p24 ELISA neutralization test required 8 days of incubation to obtain enough released virus to measure p24 antigen levels by ELISA. With few exceptions, both assays gave essentially the same level of neutralization, but the FACS assay could be performed after only 4 days of incubation.

In another study, neutralization of HIV-1 was measured in HIV-1-infected PBMCs after a single round of infection

(Mascola et al., 2002). To limit the infection of PBMCs to a single round, a protease inhibitor was included in the assay. This 2-day in vitro assay was highly sensitive and specific for detection of HIV-1-infected PBMCs. This neutralization assay provided quantitative data on the number of target cells infected and on the inactivation of infectious virus due to reaction with antibody. This assay has been proposed for use in determining the neutralizing activity of antibodies generated in response to vaccines developed from the prevention and treatment of HIV infections.

Dykes et al. (2006) developed a multiple-cycle growth competition assay to measure HIV-1 replication efficiency that uses flow cytometry to determine the relative proportion of test and reference viruses. The reporter gene products are expressed on the cell surface and they use commercially available fluorochrome-labeled monoclonal antibodies to detect each population (test or reference virus) of virus-infected cells. The assay was used to quantify the relative fitness conferred by protease and reverse transcriptase sequences containing multiple drug resistance mutations amplified from patient plasma. The FACS assay was easier and more rapid than the standard growth competition assays.

FACS analysis has been used to measure fusion between HIV-infected or transfected cells and target cells expressing CD4 and an appropriate coreceptor on their surfaces (Huerta et al., 2002). They used two different fluorescent lipophilic probes to label each cell population and flow cytometry to measure the extent of cellular fusion after coculture. They demonstrated fusion between HeLa and CHO cells in monolayers and between Jurkat and Jurkat cells in suspension lymphocyte cultures.

HCV

FACS analysis of microspheres coated with NeutraAVidin was used to capture biotinylated HCV recombinant proteins. A phycoerythrin goat anti-human IgG was used to detect antibody captured to the microspheres (McHugh, 2005). This assay was used to quantify the amount of HCV-specific antibody in clinical samples. The assay is easily performed with a flow cytometer.

FACS analysis has been used to characterize the repertoire of T-cell epitopes on the HCV core protein by measuring the major histocompatibility complex class 1 binding of decapeptides on human B-cell lines and murine spleen cells (Schweitzer et al., 2000).

Virus Receptors on Cell Surfaces

Fluorochrome-labeled virus particles were used to identify virus receptors on cell surfaces, and these interactions were detected by FACS analysis. The cell receptors for echovirus 1 and coxsackievirus A9 (CAV-9) were identified and quantified by flow cytometry (Triantafilou et al., 2001). These authors showed that echovirus 1 utilizes mainly integrin $\alpha 2\beta 1$, whereas CAV-9 utilizes $\alpha v\beta 3$ only 40% of the time, suggesting that CAV-9 also utilizes other receptors. Fluorescein-labeled poliovirus was bound to specific receptors on HeLa or U937 cells, and the number of cells with bound virus was detected by FACS analysis (Freistadt and Eberle, 2006).

MULTIPARAMETRIC ANALYSIS OF VIRUS-INFECTED CELLS

Many studies of virus-infected cells have used FACS analysis to simultaneously measure virus infection, the effect of infection on the cell cycle, and the production of cytokines in virus-infected cells. These studies show the ability of flow cytometry to measure the effects of virus infection on different aspects of cell function. Descriptions of multiparametric analyses of this body of work are available (Asmuth et al., 2005; Godoy-Ramirez et al., 2005; Adang et al., 2006; Fanning et al., 2006; Lee et al., 2007; Martinelli et al., 2007).

CONCLUSIONS

This review has demonstrated that fluorochrome-labeled antibodies and flow cytometry can be used to detect virus-infected cells in culture. The use of flow cytometry to measure the effect of virus infection on apoptosis and the cell cycle is already well established. The use of this technology for screening antiviral drugs and for drug susceptibility assays for hCMV was presented in enough detail to convince the reader of its great potential for the rapid, quantitative analysis of virus-drug interactions. Similar analyses could be performed for the detection of any virus that grows in tissue culture cells and for which antibodies to viral antigens are available for the detection of virus-infected cells. Expanded use of fluorochrome-labeled monoclonal antibodies to viral antigens and flow cytometry for detection and quantification of virus-infected tissue culture cells will save time and effort and make the diagnostic laboratory more efficient and productive.

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VIRAL PATHOGENS

II

Respiratory Viruses

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17

Acute respiratory tract illnesses are the most common health conditions affecting humans. They are also of great consequence, ranking as one of the top 10 causes of death worldwide (Mathers et al., 2006). Most of these illnesses are caused by viruses and involve the upper airways, clinically manifesting as "colds," pharyngitis, or tonsillitis. Upper respiratory tract illnesses (URTIs) are usually self-limited and relatively mild but prompt many physician visits for symptomatic relief or for complications such as sinusitis and otitis media (Monto and Sullivan, 1993; van Gageldonk-Lafeber et al., 2005; Mallia et al., 2007; Chonmaitree et al., 2008). In the United States alone, URTIs are responsible for an estimated 25 million ambulatory visits and 1.6 million emergency department visits annually (Gonzales et al., 2001).The influenza syndrome and viral lower respiratory tract illnesses (LRTIs) such as croup, bronchiolitis, and pneumonia are somewhat less frequent but are associated with higher hospitalization rates and fatalities. Virus-triggered exacerbations of asthma or chronic obstructive pulmonary disease (COPD) can likewise be severe and sometimes fatal (Papadopoulos and Kalobatsou, 2007; Varkey and Varkey, 2008). Overall, acute viral respiratory tract illnesses put a considerable strain on healthcare systems due to excess healthcare visits and hospital stays, infection control efforts, diagnostic testing, and use of medications. They also represent an important cause of work or school absence and lead to significant societal impacts and financial losses (Monto, 2004; Paramore et al., 2004; Principi et al., 2004). Annual direct and indirect costs for virus-associated acute respiratory tract illnesses are estimated at more than $10 billion per year in the United States alone.

The viruses responsible for these illnesses are collectively known as known as the "respiratory" viruses. Six "classic" respiratory viruses have been known for decades: respiratory syncytial virus (RSV), influenza virus, human parainfluenza virus, rhinovirus, adenovirus, and the coronaviruses (CoVs) OC43 and 229E. The existence of others, however, has long been suspected due to the lack of viruses recovered from over half of all specimens from symptomatic patients, even in the best of laboratories. Since 2001, many additional respiratory viruses affecting humans have been identified: human metapneumovirus (HMPV), two additional human CoVs (NL63 and HKU1), and the CoV associated with severe acute respiratory syndrome (SARS) known as the SARS-CoV, bocavirus, polyomaviruses WU and KI, and the H5N1 and H7N1 subtypes of avian influenza (AI) A (Table 1). Most of these newly described viruses had probably circulated in humans for decades prior to their discovery; the SARS-CoV and AI infections are of recent animal origin. The alarming pandemic potential and novel nature of several of these agents have created considerable interest in clinical virology among the public, medical and scientific communities, and industry.

General clinical features, epidemiology, and strategies for detection of respiratory viruses are presented, followed by descriptions of the individual RNA-containing respiratory viruses and those that contain DNA. Viruses that principally target other organs but also infect the respiratory tract, such as the enteroviruses, the herpesviruses, and the hantaviruses, are described in other chapters.

GENERAL CLINICAL FEATURES

Respiratory viruses tend to cause distinct clinical syndromes based on their tropism for a particular level of the respiratory tract (Table 2). For example, rhinoviruses are most often associated with the common cold, influenza virus with the "influenza" syndrome commonly known as "the flu," the parainfluenza viruses with croup, and RSV with bronchiolitis. These virus-syndrome associations are not completely predictable, however. Viruses can also cause symptoms anywhere along the pulmonary tract, depending on viral factors (e.g., virus subtype or inoculum), host factors (e.g., age, underlying clinical condition, nutritional variables, immune status, genetic predisposition), and environmental modifiers (e.g., exposure to air pollution). Episodes that begin as URTIs can also progress with time to involve deeper airway structures.

Infections with more than one pathogen may also blur the classic virus-syndrome associations. Influenza virus infections in the elderly, for example, can be followed by secondary bacterial pneumonia resulting in a more prolonged and severe course (Cate, 1998). *Streptococcus pneumoniae* is the most common bacterium implicated, but other bacteria can be involved, particularly where pneumococcal vaccine use is widespread (Jarstrand and Tunevall, 1975; Falsey, 2005). Recently methicillin-resistant *Staphylococcus aureus* has been associated with clusters of severe and fatal influenza LRTIs in individuals of all ages without known risk factors (Hageman et al., 2006). The relevance of such bacterium-virus coinfections is usually obvious in high-risk patients, but they are

TABLE 1 Classification of viruses that primarily infect the respiratory tract[a]

Nucleic acid type	Family	Subfamily	Genus	Species (group)
RNA	*Orthomyxoviridae*		*Influenzavirus A, B, C*	Influenza A, B, C
	Paramyxoviridae	*Pneumovirinae*	*Pneumovirus*	RSV
			Metapneumovirus	HMPV[b]
		Paramyxovirinae	*Respirovirus*	Parainfluenza virus 1, 3
			Rublavirus	Parainfluenza virus 2, 4
	Picornaviridae		*Rhinovirus*	Rhinovirus A, B, C[b,c]
	Coronaviridae		*Coronavirus*	(I) 229E, NL-63[b,c]
				(IIa) OC43, HKU1[b,c]
				(IIb) SARS-CoV
DNA	*Adenoviridae*		*Mastadenovirus*	Adenovirus B (1), B (2), C, D, E
	Parvoviridae	*Parvovirinae*	*Bocavirus*	Human bocavirus[b,c]
	Polyomaviridae		*Polyomavirus*	WU, KI[b,c]

[a]Source: International Committee on Taxonomy of Viruses (http://www.ncbi.nih.gov/ICTVdB/index.htm).
[b]Newly described.
[c]Presumptive taxonomic relationship.

of less-certain significance when illness is mild or occurs in otherwise healthy individuals (Thorburn et al., 2006; Koskenvuo et al., 2007a; Richard et al., 2008; Stensballe et al., 2008).

Virus-virus coinfections occur at rates varying from zero in several population-based studies to nearly 80% with bocavirus and the polyomaviruses (Greensill et al., 2003; Jennings et al., 2004; van Woensel et al., 2006; Allander et al., 2007b; Bialasiewicz et al., 2008). This wide range is due in large part to the technical limitations of detecting multiple viruses simultaneously, particularly by culture (Landry, 1994; Waner, 1994). In some studies, viral coinfections appear to be more severe than mono-infections and a potential predictor of outcomes. For example, a recent evaluation of infants with severe bronchiolitis noted that coinfected infants had an almost threefold-higher risk of intensive care unit admission than those with mono-infections (Richard et al., 2008). This association is not universally noted (Norja et al., 2007). Interestingly, rhinoviruses, adenoviruses, and bocavirus are the most frequent participants in dual infections (Jennings et al., 2004; Tsolia et al., 2004; Choi et al., 2006; Allander et al., 2007b; Regamey et al., 2008). Further studies are needed to clarify the significance of virus-virus coinfections.

EPIDEMIOLOGY

Geographic Distribution and Seasonality

Viral respiratory tract infections occur worldwide. In countries with definite winters, annual epidemics of RSV, influenza virus, and HMPV occur with reasonable regularity during the cooler months of the year. Influenza virus tends to produce a sharp annual peak of respiratory illness lasting 6 to 8 weeks, whereas the peak of RSV illness tends to be broader with a median duration of 15 weeks in each locale. The onset of RSV outbreaks varies considerably year to year and even between communities in close proximity, although there are general regional patterns. For example, the southern region of the United States has an RSV season that begins approximately 6 weeks earlier and lasts 3 weeks longer than the RSV season in the Midwest United States (Panozzo et al., 2007). Alaska and Hawaii have atypical RSV epidemic on/off times. One recent study suggested that the RSV season now ends earlier due to global climate change (Donaldson, 2006). HMPV can be detected year-round but usually peaks in late winter to spring, coincident with or slightly later than RSV. Considerable variation in HMPV detection rates from season to season at the same locale is also noted (Williams et al., 2006; Weigl et al., 2007).

TABLE 2 Relative importance of major respiratory viruses in upper and lower respiratory tract diseases

Virus	Importance in[a]:				
	Common cold	Flu/flu-like illness	Croup	Bronchiolitis	Pneumonia
Influenza	+++	++++	+	+	++++
RSV	+++	+	++	++++	++++
HMPV	++	+	+	++++	++++
Parainfluenza	+++	+	++++	+++	++++
Rhinovirus	++++	++[b]	+	–	++[b]
CoV 229E, OC43	+++	+	+	+	+
CoV NL-63[b]	++	+	+++	+++	++
Adenovirus	+++	++	+	–	++++

[a]+ to ++++, minimal to major importance; –, no or negligible importance.
[b]New associations, frequency not yet established.

Seasonality of the four parainfluenza virus serotypes is type specific and interactive. Parainfluenza virus type 1 causes large biennial epidemics in the autumns of odd-numbered years and is sometimes accompanied by type 2 but in lower quantities. Circulation of parainfluenza virus type 3 is more endemic, with springtime peaks at some locales. A second smaller autumn peak of type 2 or 3 sometimes appears when type 1 is not prominent. Parainfluenza type 4 is rarely detected, so its seasonality is not clearly defined (Fry et al., 2006; Weigl et al., 2007). Respiratory adenoviruses, rhinoviruses, and CoVs OC43 and 229E are more endemic in their circulation, although increased activity often occurs in the cooler months. Outbreaks of the CoVs tend to occur every 2 to 4 years in an alternating pattern. Infections due to CoV 229E tend to be more widespread, whereas OC43 is predisposed to more localized outbreaks (Monto, 2002; Wong et al., 2008). The temporal pattern of four common respiratory viruses detected in symptomatic U.S. children is depicted in Fig. 1.

Despite the relative regularity of these overall patterns, the intensity of each epidemic and onset and cessation dates can vary, even among communities in close proximity. Current and previous temporal and geographic patterns of most classic respiratory viruses in the United States are now available electronically on the National Respiratory and Enteric Virus Surveillance System (NREVSS) website, http://www.cdc.gov/surveillance/nrevss/. The seasonality of most newly described respiratory viruses remains mostly investigational. Even less is known about respiratory virus circulation in regions with tropical or subtropical climates. New information is emerging, however, due to concern that viruses with pandemic potential may originate from these locales. In some of these warmer climes, influenza and RSV persist year-round, with periodic increases in circulation seemingly related to changes in temperature or rainfall (Shek and Lee, 2003; Stensballe et al., 2003).

Why respiratory virus epidemics occur seasonally remains a puzzle. Factors favoring virus survival, such as low temperature and low relative humidity are probably important, but seasonal changes in host physiology and crowding may be contributory (Dowell, 2001; Cannell et al., 2006; Lowen et al, 2007). A related question, where do respiratory viruses persist between outbreaks, remains unanswered even for well-studied pathogens like RSV and influenza virus. One theory holds that many seasonal respiratory viruses persist from the preceding season in chronically ill individuals and are maintained by low-level person-to-person transmission until environmental and social conditions facilitate more efficient spread. Intercontinental travel bringing viruses such as influenza into populations with waning herd immunity, followed by contiguous spread, is another proposed mechanism. No one explanation seems to be completely satisfactory, even for a single virus (Dowell, 2001; Stensballe et al, 2003).

Transmission

Respiratory virus transmission has assumed great significance recently due to concerns about how viral pandemics spread. There are three major routes of respiratory virus transmission: direct contact with secretions, direct inhalation of large (≥5 μm) droplets or small (<5 μm)-particle aerosols, and indirect contact with inanimate objects (fomites) contaminated with respiratory secretions. All routes of transmission probably occur for every respiratory virus, but among them, indirect contact is probably the most important (Boone and Gerba, 2007; Brankston et al., 2007). The SARS-CoV, AI, and perhaps bocavirus and the polyomaviruses may be spread by nonrespiratory routes as well.

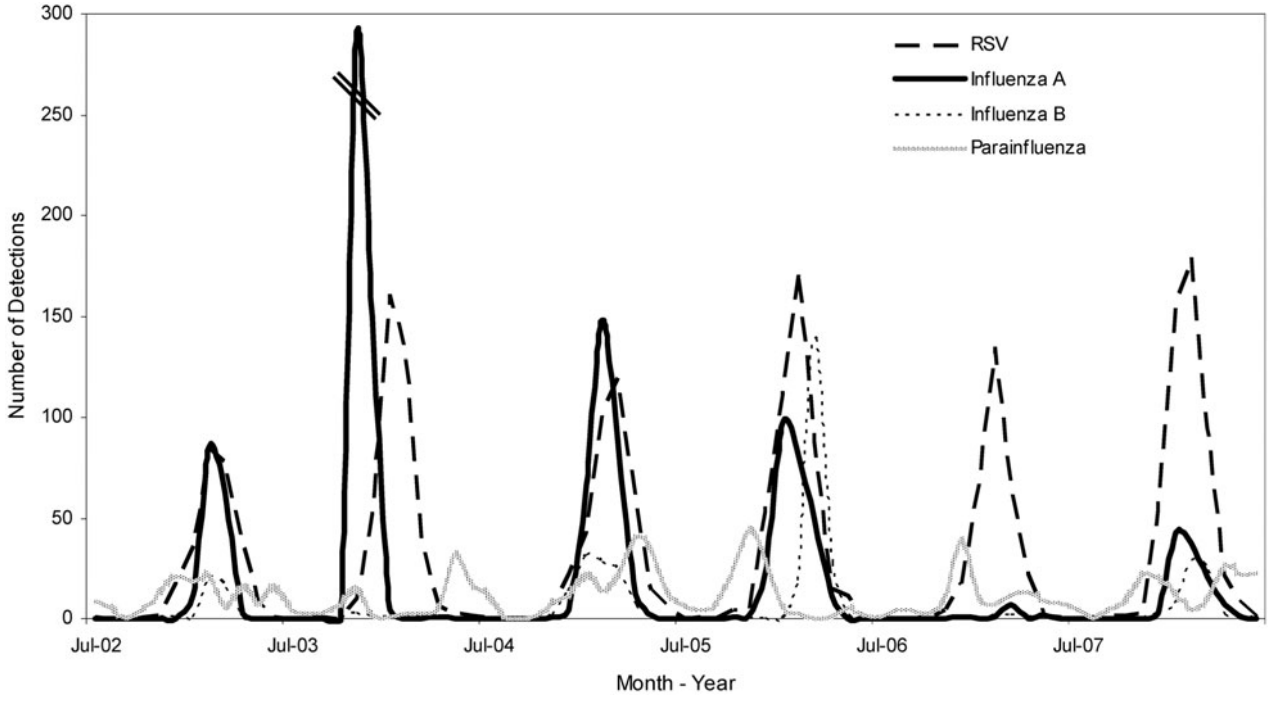

FIGURE 1 Temporal patterns of four common respiratory viruses detected over a 6-year period by The Children's Hospital Virology Laboratory, Denver, CO. Numbers of detections are as indicated, except during a large outbreak of influenza A in November and December of 2003 when approximately 950 and 400 specimens were positive for influenza A, respectively.

Different routes of respiratory transmission are not mutually exclusive, and their relative contribution can be difficult to unravel in natural settings. Both droplets and aerosols can be propelled by a sneeze or cough. The droplets fall onto fomites, can deposit directly onto the mucosa of individuals in the immediate environment, or shrink by evaporation into droplet nuclei. Aerosols and droplet nuclei can then float over long distances to be inhaled into the lower airways, where they rehydrate and regain infectivity. Contaminated fomites can remain infectious for hours to days, providing a source of virus for hands that subsequently auto-inoculate the eyes or mouth. Persistence of virus on fomites is related to virus type and inoculum, nature of the surface, temperature, humidity, and perhaps other factors (Bean et al, 1982; Duan et al., 2003; Lowen et al., 2007). For example, infectious RSV is recoverable from skin for 20 minutes, from cloth or paper tissues for 45 minutes, and from solid surfaces, such as stethoscopes, for up to 6 h (Hall et al., 1980; Blydt-Hansen et al., 1999). In contrast, influenza virus is stable on surfaces for up to 2 days (Bean et al., 1982).

Many means can interrupt transmission of respiratory viruses. Vaccination and antiviral therapy are highly effective at limiting the spread of influenza virus, but these approaches are not available for other respiratory viruses. A recent, large meta-analysis concluded that simple and low-cost physical interventions such as frequent hand-washing, wearing a mask, and isolation of potentially infected patients significantly reduce transmission of many respiratory viruses, including influenza (Jefferson et al., 2008). These basic practices appear to be most effective when aimed at young children due to their frequent infections, shedding of high virus titers, and low awareness of personal hygiene. There was no benefit of virucidals over good hand-washing in this analysis. Two types of masks were used during the SARS outbreak and continue to be evaluated for other respiratory virus pandemics (Loeb et al., 2004; Tellier, 2006). One mask blocks 95% of small-particle aerosols and larger droplets and is known as the N95 mask. The other is a simple surgical mask. Objective data to recommend one over the other remain limited, so both are mentioned in the current U.S. plan for management of pandemic influenza. Other effective physical interventions called "respiratory hygiene" or "cough etiquette" involve simple but effective measures such as coughing or sneezing into the elbow and disposing of tissues containing respiratory secretions in waste receptacles.

Susceptible Populations

All individuals are susceptible to acute viral respiratory tract illness. Culture-based surveillance studies of the classic respiratory viruses in U.S. families during the 1960s and 1970s, however, demonstrated that this burden varies considerably by age. Infants under 6 months of age are relatively spared. Infections then peak in 2 to 5 year olds and slowly decline throughout adulthood. Children in daycare or school and adult caregivers of young children experience more respiratory virus infections than persons of comparable age in less-crowded settings (Monto and Sullivan, 1993). These conclusions are supported by more recent studies using comprehensive molecular assays. In one representative study of individuals seeking medical attention for respiratory symptoms in The Netherlands, approximately 1,900 respiratory virus-associated illness episodes occurred per 10,000 person years in the 0- to 4-year-old age group, 600 per 10,000 person years in 5- to 24-year-olds, and 400 per 10,000 person years in individuals 25 years of age and older. Persons 65 years of age and older had the same or slightly lower rates of illness than the general adult population (van Gageldonk-Lafeber et al., 2005).

Most LRTIs, and therefore the highest rates of morbidity and mortality, occur in individuals at both extremes of life. Children are the most vulnerable. In the United States alone, over 5 million children less than 18 years of age acquire viral LRTIs, and over 500,000 are hospitalized each year (Henrickson, 1998). Children under 2 years of age, particularly infants less than 2 months old, are at highest risk, primarily due to the small caliber of their airways and immunological naiveté. Predictably, defects or chronic disease of the cardiopulmonary, neurologic, or immune systems also predispose children to adverse outcomes (Simoes, 2003; Henrickson et al., 2004). Other contributing factors may include lack of breastfeeding, smoking in the home, nutritional status, and a family history of atopy (Simoes, 2003; Henrickson et al., 2004; Bradley et al., 2005; Heymann et al., 2005). The bronchopulmonary dysplasia that can develop following premature birth confers continued risk for significant morbidity due to acute viral respiratory tract infections during the first year of life and sometimes beyond (Broughton et al., 2007). Children in resource-poor settings are especially susceptible to severe LRTIs due to frequent reinfections (Nokes et al., 2008). There is likely an additive effect when multiple risk factors are present.

The elderly are likewise susceptible to URTIs that progress to serious lower airway disease. The frail elderly are at highest risk. Influenza is their most common pathogen, causing 25,000 to 60,000 excess deaths in people aged 65 years or over in the United States each year (Greene et al., 2006). RSV is also a significant problem and is probably responsible for as many as 10,000 deaths due to LRTIs in the U.S. elderly annually (Falsey, 2007). Other respiratory viruses, even rhinoviruses, can cause significant morbidity and mortality in older individuals as well (Falsey and Walsh, 2006; Hicks et al., 2006; Boivin et al., 2007). Age-specific declines in respiratory and immune function, reduced viral clearance, and perhaps poor oral hygiene likely contribute to heightened susceptibility in this age group, although further research in this area is needed (Abe et al., 2006; Falsey and Walsh, 2006).

Previously healthy working adults are usually considered at low risk of viral LRTIs. Serious infections in this population are probably more frequent than generally realized but are often missed due to lack of clinical recognition. Technical issues, such as the need for lower airway specimens and insensitivity of antigen assays in adults compared to children, also contribute to underdiagnosis (Falsey and Walsh, 2000; Casiano-Colon et al., 2003; Babcock et al., 2006). Influenza virus is probably the most serious viral pathogen in this age group, although RSV caused LRTI in 5 to 10% of otherwise healthy adults in one prospective study (Falsey and Walsh, 2000; Angeles et al., 2006; Jansen et al., 2007).

Other groups at high risk of adverse outcomes include individuals with underlying pulmonary abnormalities. For example, it is estimated that 40 to 60% of exacerbations in patients with COPD are triggered by respiratory virus infections (Seemungal et al., 2001; Beckham et al., 2005). Similarly, many pulmonary exacerbations in patients with cystic fibrosis are associated with acute viral respiratory tract infections (Wat et al., 2008). Other susceptible populations include individuals with technology-dependent breathing disorders, cardiac malfunction, neurologic or neuromuscular disease, or hemoglobinopathies (Henrickson et al., 2004; Panitch, 2004).

Outcomes for immunocompromised patients vary by underlying diagnosis. Hematopoietic stem cell transplant

(HSCT) recipients are at the highest risk. Parainfluenza virus, RSV, and influenza virus infections tend to be most severe in this group, although almost any virus infection can be problematic (La Rosa et al., 2001; Englund et al., 2006; Gutman et al., 2007). Progression from viral URTIs to pneumonia in the first 100 days post-HSCT has been reported in 18 to 44% of cases, with fatalities in 25 to 45% of cases within 30 days of a pneumonia diagnosis (Nichols et al., 2001b; Nichols et al., 2004b). Somewhat lower mortality rates have been observed recently due to better supportive care, testing and treatment of patients with milder illnesses, and active surveillance. Risk factors for poor outcomes include time of year, older age, lack of engraftment or lymphopenia, relapse of malignancy, presence of graft-versus-host disease, and pretransplantation viral infection (Peck et al., 2004; Kim et al., 2007). Shedding of virus can be prolonged in symptomatic and asymptomatic HSCT patients, leading to progressive airflow obstruction and spread of virus to others (van Kraaij et al., 2005; Debiaggi et al., 2006; Erard et al., 2006; Peck et al., 2007).

The impact of respiratory virus infections on recipients of solid-organ transplant (SOT) is less well studied. A spectrum of illness ranging from mild URTIs to life-threatening pneumonias is reported, with some acute respiratory virus infections triggering graft rejection. The mortality rate due to these infections is lower than in HSCT patients, except for recipients of lung transplants. Respiratory virus infections involving the lower airways in lung transplant patients can trigger acute rejection or a special form of chronic rejection known as bronchiolitis obliterans (Khalifah et al., 2004; Kumar et al., 2005; Kaiser et al., 2006). Adult and pediatric cancer patients are also at increased risk for complicated respiratory virus infections. Some fatalities are reported, especially during periods of intensive chemotherapy (Chemaly et al., 2006; Mendoza Sanchez et al., 2006).

Individuals with human immunodeficiency virus (HIV) infection are at lower risk of serious respiratory virus infections than other immunocompromised patients, but most studies find poor outcomes due to acute respiratory virus infections in HIV-infected individuals compared to uninfected counterparts (Madhi et al., 2000; Madhi et al., 2002; Mendoza Sanchez et al., 2006). Dissecting out the contribution of other concurrent illnesses in these complex patients, however, has proven difficult. The prevailing view is that respiratory infections due to nonviral pathogens are more problematic than respiratory virus infections in these individuals. A recent PCR-based study of lower airway tract specimens from HIV-infected patients with respiratory symptoms, however, found respiratory viruses in approximately 20% of cases, of which 63% had only a respiratory virus. This finding suggests that the importance of respiratory viruses in this population is underestimated (Garbino et al., 2008). Further studies using molecular methods, rather than classic techniques, may be informative. Prolonged shedding of respiratory viruses occurs in individuals, especially children, with HIV, facilitating repeated infections and nosocomial spread (Madhi et al., 2007).

Overall, respiratory virus infections in immunocompromised patients tend to be of long duration, progress from URTIs to pneumonia in a higher proportion of cases, and are associated with more adverse outcomes than similar illnesses in otherwise healthy persons of comparable age. These infections can also pave the way to localized or disseminated bacterial disease. An increase in serious respiratory virus infections in upcoming years is projected due to the expanding number of immunocompromised and elderly individuals worldwide.

DIAGNOSIS

Most individuals with respiratory virus infections do not seek or require a specific diagnosis, yet establishing a viral etiology rapidly is beneficial in many circumstances. Diagnosis usually requires laboratory testing, since clinical characteristics and seasonality do not accurately discriminate between causative agents. A frequent reason for testing is to detect influenza virus, the only respiratory virus for which antivirals are approved and available. Treatment may be important in other special circumstances. For example, cidofovir may reduce the mortality associated with adenovirus infections in transplant patients, ribavirin may be useful against RSV in immunocompromised patients, and corticosteroids may reduce recurrent wheezing following rhinovirus infections but not those associated with RSV (Jartti et al., 2006; Kim et al., 2007; Anderson et al., 2008). Documented nontherapeutic advantages of rapid viral diagnosis are also many. They include better antibiotic stewardship, prevention of nosocomial infections, reduced laboratory use, improved patient and physician satisfaction, reduced lengths of stay, and lower hospital costs (Woo et al., 1997; Barenfanger et al., 2000; Byington et al., 2002). At some centers, HSCT is delayed if a respiratory virus is detected prior to pretransplant conditioning (Peck et al., 2004).

Approaches to laboratory detection of respiratory viral infections are viral culture, detection of viral nucleic acid or antigen, and serology. Viral culture is slow, but it is broad minded and can be useful when an isolate is needed for further characterization. In contrast, detection of viral antigen or nucleic acids can provide a specific diagnosis in less than a day, even when there is no viable virus in a specimen. Serology plays little role in the diagnosis of acute respiratory tract disease because other methods are more rapid, but it can be helpful for epidemiologic purposes or to establish a viral diagnosis retrospectively. Results can be difficult to interpret in immunocompromised persons due to failure of antibody production and in infants due to the presence of maternal antibody.

Optimal detection depends on the proper timing of specimen collection, type and quality of sample, patient age, and the specific assay. In most acute viral respiratory illnesses, maximum virus shedding occurs in the first 3 to 5 days after onset of illness. Therefore, specimens should be collected as soon as possible after patients seek care. Virus shedding can be detected in some cases for up to 2 weeks if sensitive assays are used. Most viral LRTIs also involve the upper airways, so specimens from nose or throat are often informative and are more easily obtained than from the lower airways. Specimens from the posterior nasopharynx (NP) are particularly desirable because they usually contain high titers of virus and large quantities of infected cells, particularly for immunofluorescence (IF). The highest yield of virus and infected cells is usually obtained from NP aspirates or washes, which are often sent from pediatric patients because they tolerate such collection. Older patients usually prefer NP swabs, which can be satisfactory if carefully collected. Most studies, however, report lower detection rates using swabs than using aspirates or washes, particularly from adults (Landry et al., 2000; Landry and Ferguson, 2000). The newly developed flocked swabs (Copan Diagnostics, Murietta, CA) which are specifically designed to collect and release cellular material

may overcome this limitation (Daley et al., 2006). Throat swabs can recover influenza virus or adenovirus by culture or rapid immunoassay (IA) but are unsuitable for IF because they contain mostly squamous cells, which do not support respiratory virus replication. Combining throat and NP swabs in the same tube can improve virus detection by culture (Weinberg et al., 2004). Sputum is not widely used because of its mucoid nature and low respiratory cell content but can yield virus by culture and in molecular assays (Kimball et al., 1983; Falsey, 2007). Recovery is usually highest from children who tend to shed virus in higher titers and for longer periods than adults. Detection of virus in adults, in immunocompromised patients, and in patients with LRTIs can be improved by use of lower airway specimens such as bronchoalveolar lavage fluid, bronchial washes, tracheal aspirates, and lung tissue (Englund, 1996).

Respiratory virus culture is necessarily complex due to the many virus types and their varying growth requirements. Optimal culture for the widest virus array usually requires a combination of primary cells and heteroploid cell lines. Commercially prepared cells growing in tubes are most often used. A common selection of cells includes primary monkey kidney cells, which replicate parainfluenza and influenza viruses; human fibroblasts, such as MRC5, for rhinoviruses; and cell lines such as A549 or Hep-2 for RSV and adenovirus. Use of roller drums may hasten the appearance of virus, which sometimes takes as long as 10 days or longer. Although most viruses will replicate at 35 to 37°C, duplicate tubes should be incubated at 33 to 34°C to improve isolation of rhinoviruses and influenza virus. Serum-free medium is needed for optimum recovery of parainfluenza and influenza viruses. These viruses often fail to produce cytopathic effect (CPE), so hemadsorption with guinea pig red blood cells is often used to reveal their presence, followed by identification by IF or other methods. Even the best routine culture systems, however, cannot detect the CoVs, polyomaviruses, bocavirus, and some rhinoviruses.

Centrifugation-enhanced rapid culture can reduce the time to detect RSV, parainfluenza virus types 1 to 3, influenza A and B, and adenovirus to 1 to 3 days. This method typically involves centrifugation of specimens onto cells growing on coverslips in shell vials or in microwell plates, followed by IF to detect viral antigen (Espy et al., 1986; Espy et al., 1987; Rabalais et al., 1992). Individual monoclonal antibodies can be used, but pooled reagents are popular because different respiratory viruses can be detected simultaneously. Subsequent staining of reserved cells using individual reagents can then identify the virus present. Some commercially available pools contain antibodies labeled with different fluorophores of overlapping spectra which permit immediate identification of individual viruses. Sensitivities of rapid culture vary widely among studies, but generally 60 to 85% of specimens positive in tube culture for the viruses listed above can be recovered. A sizeable proportion of HMPV-positive specimens can also be detected (Landry et al., 2005). A major drawback of this method is that rhinoviruses, CoVs, and most newly discovered respiratory viruses cannot be detected. The requirement for multiple cell types can be reduced by using a commercially available mixture of mink lung and A549 cells (R-Mix; Diagnostic Hybrids, Athens, OH) which can detect influenza virus, parainfluenza virus types 1 to 4, and the SARS-CoV (St. George et al., 2002; Gillim-Ross et al., 2004). Recovery of RSV and adenovirus, however, is not optimal with these cells (LaSala et al., 2007). A newer mixed-cell formulation (R-Mix Too) contains Madin-Darby canine kidney (MDCK) and A549 cells, does not support SARS-CoV, and is recommended for clinical laboratories if this virus begins to circulate again.

Assays that detect viral antigen are widely used because they can provide results in a clinically relevant timeframe. They are especially useful in hospital settings for rapid triage of vulnerable patients, with more complex or slower testing reflexively performed on virus-negative specimens. The simplest of such methods are the membrane-based, single-use, rapid IA cassettes. Most rapid IAs can be performed in less than 30 min with minimal hands-on time and are suitable for personnel with little formal laboratory training; some are also approved for near-patient testing. Microtiter plate formats are available as well and are often preferred by larger-volume laboratories. A major disadvantage is that rapid IAs are only available for RSV, adenovirus, and detection and differentiation of influenza A and B (Fujimoto et al., 2004; Borek et al., 2006; Smit et al., 2007). Sensitivities also vary widely, with reported values ranging from 45 to 95% for influenza and 60 to 90% for RSV. The highest values are obtained when specimens from pediatric patients are tested (Leland and Ginocchio, 2007). Specificities are likewise variable but are usually greater than 97%, yielding positive predictive values that are reasonably high when the target viruses are circulating but are unacceptably low at other times. This limitation has prompted the U.S. Centers for Disease Control and Prevention (CDC) to recommend restricting use of influenza IAs to periods of peak virus activity and confirmation of all positive results at other times by IF, culture, or an assay for viral nucleic acid (Grijalva et al., 2007).

When suitably trained personnel and equipment are available, antigen detection by IF is often preferred over rapid IAs because many viruses, including RSV, influenza A, influenza B, parainfluenza virus types 1 to 3, adenovirus, and HMPV, can be identified. Some of the same monoclonal antibodies available for centrifugation culture can be utilized (Landry and Ferguson, 2000; Landry et al., 2008). Results can be provided in 30 min to an hour, and many mixed infections can be identified. The characteristic patterns and intensity of IF staining generally ensure higher specificity than IA, and specimen adequacy can be evaluated. The overall sensitivity of IF is approximately 80% compared to culture but varies by virus (Leland and Ginocchio, 2007). The highest sensitivities (80 to 95%) are generally achieved with RSV (Fong et al., 2000) and the lowest (40 to 60%) with adenovirus (Robinson and Echavarria, 2007). Preparation of slides by cytocentrifugation prior to staining can reduce the percentage of inadequate specimens and increases assay sensitivity (Landry et al., 2000).

Molecular assays, also known as nucleic acid amplification tests (NATs), permit the widest array of respiratory viruses to be identified, including the classic respiratory viruses and newly described viruses not detectable by other means. NATs are also useful when specimen quality is compromised or if samples are collected late in the course of illness. In most recent studies, detection rates by this technology are equivalent to or higher than those achievable by antigen assays or viral culture, and coinfections are more frequently observed (Jennings et al., 2004; Fox, 2007; Wright et al., 2007). Most NATs for respiratory viruses utilize PCR, but other chemistries are also available. Commercial systems for nucleic acid extraction and NAT are now available that standardize, speed, and simplify the detection of respiratory viruses individually or in panels. Multiplex assays are especially applicable for testing children or immunocompromised patients because of the vulnerability of these subjects to a wide range

of viruses (Erdman et al., 2003; Kuypers et al., 2006). In 2008, several sensitive and specific multiplex PCR-based NATs for respiratory viruses were approved for diagnostic use in the United States, with others soon to follow suit (Legoff et al., 2008; Pabbaraju et al., 2008). Although the widespread use of NAT is still limited to larger facilities or academic centers due to the need for specialized expertise, assay validation, and specially designed laboratory space, acceptance is expected to increase significantly in the near future.

Caution remains warranted, however, for several reasons. Although NAT often targets highly conserved regions in viral genomes or uses degenerate primers, false-negative results can occur from even small mutations in regions of primer or probe binding. This issue is especially problematic with viruses with multiple species or types. False-positive results can also be generated by cross-contamination of specimens or individual amplification reactions, so extreme care is required during specimen handling, nucleic acid extraction, and testing. Persistence of viral nucleic acid for prolonged periods of time may also cloud assessment of causality or the significance of coinfections. Despite these limitations, NAT is now emerging as the new gold standard for respiratory virus detection and as a major tool for discovery of novel pathogens.

VIRUS BIOLOGY, EPIDEMIOLOGY, DIAGNOSIS, PREVENTION, AND THERAPY

Influenza Virus

Of all the viruses that infect the respiratory tract, influenza causes the largest number of serious acute illnesses. Most of these episodes manifest clinically as a triad of fever, myalgias, and pharyngitis known as "influenza" syndrome. Seasonal epidemics of influenza in temperate climates can involve 10% or more of the entire population each winter, causing more than 200,000 hospitalizations and 30,000 deaths in the U.S. annually (Thompson et al., 2004). The toll can be much higher during a pandemic. Hospitalization rates are the highest in young children and in persons over 65 years of age, with over of 90% of fatalities occurring in the elderly. The continued aging of the U.S. population has caused a steady increase in the medical burden of influenza over the past 2 decades (Glezen, 2004). In addition, an extensive epizootic of AI is intermittently crossing into humans, with apparently high mortality and occasional human-human spread. On a global scale, the human consequences of AI remain minor. Continued interspecies transmission and global travel, however, increase the likelihood that an avian strain with major pandemic potential will emerge (Johnson and Mueller, 2002). These two issues have focused considerable attention on the diagnosis and control of influenza virus infections in recent years.

Influenza viruses belong to the *Orthomyxoviridae* family. There are three genera with eponymous members infecting humans: influenza virus A, influenza virus B, and influenza virus C (Table 1). Their name stems from the Latin *influentia* which means "epidemic" because outbreaks of influenza were once thought to be under astrological or occult influence. Influenza A and B cause the greatest proportion of human infections, with influenza A responsible for most seasonal epidemics and all known pandemics. Influenza A was initially described in the 1920s as a transmissible agent in human mucus capable of infecting swine and was recognized as a virus in the mid-1930s. Influenza B and C were described as distinct pathogens more than a decade later. Influenza A naturally infects many bird species, humans, and other mammals, including swine, seals, felines, and horses. Infections with influenza B and C are generally restricted to humans (Osterhaus et al., 2000).

Influenza virions are enveloped, spherical, and pleomorphic in size and shape, ranging from 80 to 120 nm in culture (Lamb and Krug, 2001). Within each particle is a single-stranded, negative-sense, segmented RNA genome. Influenza A and B have 8 RNA segments, whereas influenza C has 7 segments. The segments are 0.8 to 2.5 kb in length, resulting in a total genome length of 10 to 14.6 kb. Structural proteins include the nucleoprotein (N), which binds to the various RNA segments and is the major component of the helical nucleocapsid, three RNA polymerase complex proteins (PB1, PB2, and PA) that function in virus replication and transcription, and the matrix (M1) protein, which is interposed between the N and viral envelope. In influenza A, a second matrix (M2) protein forms an ion channel across the envelope and is important in virus uncoating. Radiating from the envelope of influenza A and B are 10- to 14-nm-long glycoprotein spikes of two types. The rod-shaped spikes of the viral hemagglutinin (HA) permit attachment of virus to sialic acid-containing cellular receptors and help initiate infection. The viral HA molecules also bind to sialic acid residues on red blood cells of certain species, causing hemagglutination, which is a useful diagnostic tool. Most human influenza viruses bind preferentially to sialic acid receptors attached to an adjacent galactose residue in an α2,6 configuration. The mushroom-shaped spikes of neuraminidase (NA) facilitate release of mature virions from infected cells and may assist in virus movement through mucus to adjacent cells. Influenza C lacks NA, but it possesses a glycoprotein with combined hemagglutination, esterase, and fusion activities.

The three influenza genera can be discriminated by antigenic differences in the M1 protein and nucleoprotein. Influenza A is further classified into subtypes, based on characteristics of its HA and N molecules. Sixteen antigenically distinct HA and 9 N molecules are known (Fouchier et al., 2005). Viruses with all H and N types can be recovered from birds, but only viruses with H1, H2, or H3, and N1 or N2 currently circulate in humans (Wright and Webster, 2001). The most common influenza A subtypes circulating globally from 1994 to 2005 were H3N2 (90.6%), H1N1 (8%), and H1N2 (1.1%) (Bright et al., 2005). During the 2007 to 2008 influenza season in the United States, approximately 74% of submitted influenza A isolates typed as H3 (Centers for Disease Control and Prevention, 2008). Higher mortality is usually observed in seasons when influenza A (H3N2) predominates than in seasons with predominant influenza A (H1N1) or influenza B (Greene et al., 2006).

Influenza viruses have evolved efficient mechanisms to promote antigenic variability. The most continuous process is "antigenic drift," which occurs with all 3 influenza virus types. Antigenic drift is caused by sequential point mutations in HA or N genes that arise during viral RNA replication and immune selection, giving rise to new strains. Antigenic shift can render the host more susceptible to infection if existing antibodies incompletely neutralize the variant. The rate of antigenic change is greatest for influenza A, with multiple major and minor strains cocirculating at any given time (Ghedin et al., 2005). An individual influenza strain is uniquely identified by its host of origin, geographic location of first isolation, strain number, and year of isolation; the antigenic subtype of influenza A is given parenthetically. The animal origin of a nonhuman strain is also given. For example, a human influenza A strain in the 2008 to 2009 vaccine is A/Brisbane/10/2007 (H3N2), whereas an avian strain in

the current influenza A epizootic is A/quail/Vietnam 36/2004 (H5N1). Influenza B undergoes antigenic change very slowly and is not categorized into subtypes. Currently, two lineages of influenza B (Yamagata and Victoria) are circulating worldwide (Centers for Disease Control and Prevention, 2008). Antigenic shift requires annual reformulation of the influenza vaccine to ensure maximum efficacy against currently circulating strains of influenza A and B.

The second mechanism whereby influenza virus antigens evolve is "antigenic shift." Antigenic shift is a more abrupt and dramatic change that occurs when a circulating influenza A strain acquires different H or N genes, resulting in a completely new strain or subtype. Antigenic shift results from the segmented nature of the influenza genome, which permits gene reassortment during a mixed infection. The novel progeny that result are known as reassortants and are often found in nature, particularly among birds. Reassortants between human and AI viruses have been the cause of most pandemics. For example, the Asian flu pandemic of 1957 was caused by a reassortant derived from the circulating human H1N1 influenza A strain and the H2, N2, and PB2 genes of AI (Kawaoka et al., 1989; Schafer et al., 1993). Swine often act as intermediaries in the reassortment process because their respiratory epithelium can be infected by influenza viruses of avian and human origin. Reassortment is also described for influenza B but has limited clinical relevance (Chi et al., 2005). Antigenic shift also occurs when a highly pathogenic animal influenza subtype crosses into humans in toto. Pandemics, however, require not only influenza strains that fail to be recognized by existing antibodies but also efficient human-to-human transmission. Pandemics occur infrequently, but the associated mortality can be devastating. Five influenza A pandemics have occurred in recent history (Table 3). Most originated in Asia and involved H1, H2, or H3. The most extensive and lethal was the devastating "Spanish flu" pandemic of 1918 to 1919, which resulted in 50 million deaths worldwide. Over 500,000 of these fatalities were in the United States. The cause of the Spanish flu is now thought to be antigenically novel influenza A (H1N1) of completely avian origin which, after a period of adaptation in humans, emerged in pandemic form (Reid et al., 2004). Survivors of a pandemic typically acquire immunity to the new virus, which gradually evolves into a seasonally circulating strain. Indeed, currently circulating strains of influenza A (H3N2) are considered descendents of the virus that caused the "Hong Kong flu" pandemic of 1968.

Seasonal influenza epidemics are typically preceded by an abrupt increase in school absenteeism because children are the initial vectors for infection of adults (Reichert, 2002). Attack rates in children are 10 to 40% each year, with approximately 1% of cases requiring hospitalization. Influenza then disperses throughout the community, with nosocomial outbreaks reported in nursing homes and other closed settings. Attack rates as high as 70% can occur in the general population from common-source exposures in a closed space (Moser, 1979). Global surveillance of such epidemics has recently been expanded to improve vaccine composition and provide an early warning system of viruses with pandemic potential. The current U.S. influenza surveillance program of the CDC includes monitoring of excess pneumonia and influenza deaths above a calculated "epidemic threshold," tallies of pediatric deaths, assessment of weekly virology data, and typing of influenza virus isolates submitted by reference laboratories (Thompson et al., 2006). The World Health Organization (WHO) coordinates a similar program in over 80 other countries. Access to current data from both organizations is available at the CDC website at http://www.cdc.gov/flu/weekly/fluactivity.htm.

Influenza is transmitted person to person primarily by inhalation of large-particle droplets. Spread by fomites or small-particle aerosols is also documented, but their relative contribution to transmission of disease remains uncertain (Tellier, 2006; Brankston et al., 2007). The typical incubation period is 1 to 4 days (average, 2 days). Onset is usually abrupt, unlike other respiratory virus infections. The constellation of the classic "influenza" syndrome includes fever, which is present in over 90% of cases; respiratory symptoms such as nonproductive cough, sore throat, and rhinitis; and systemic symptoms such as malaise, myalgia, and headache (Nicholson, 1992). In uncomplicated cases, the illness usually resolves in about a week, although cough and malaise can linger for 2 weeks or longer.

A protracted course and complications of influenza are more frequent in high-risk patients. Virus shedding begins before onset of symptoms and is greatest in the first week of illness. It can also be detected beyond 10 days in some children, in influenza B infections, and for weeks to months in immunocompromised patients (Frank et al., 1981; Weinstock et al., 2003). Recovery is associated with the appearance of virus-specific immunoglobulin M (IgM) and IgA antibodies peaking about 2 weeks after infection and an IgG response that develops 4 to 7 weeks thereafter. Innate immunity is also important for recovery, but the factors involved and their relative contributions remain investigational (Sladkova and Kostolansky, 2006).

Influenza in children can be a significant illness, yet it is often overlooked due to a more variable clinical picture. Presentations can vary from a sepsis-like illness with high fevers and febrile seizures, fever with few respiratory tract symptoms, URTI, croup, or bronchiolitis to a classic influenza-like illness (Wang et al., 2003b; Ohmit and Monto, 2006). Nausea, vomiting, diarrhea, and conjunctivitis may also occur.

TABLE 3 Recorded influenza pandemics

Year(s) of occurrence	Common name	Subtype	Likely source	No. of deaths	
				Worldwide	United States
1889–1890	Asiatic flu	Perhaps H2N2	Unknown	10 million	Unknown
1918–1919	Spanish flu	H1N1	Human-adapted AI	25–50 million	More than 500,000
1957	Asian flu	H2N2	Human-avian reassortant	More than 1 million	70,000
1968	Hong Kong flu	H3N2	Human-avian reassortant	More than 1 million	34,000
1977	Russian flu	H1N1	Perhaps from 1950s frozen stock	Few	Few

Indeed, the positive predictive value of a classic clinical definition of influenza (fever and cough) during the wintertime can be as low as 17% among children treated as outpatients and 28% among hospitalized children compared to 50 to 88% in adults (Boivin et al., 2000; Monto et al., 2000; Babcock et al., 2006). Likewise, influenza in the elderly can produce symptoms so nonspecific that positive predictive values as low as 30% in this population are reported (Govaert et al., 1998). Low values also occur when virus activity is slow, as in the beginning and end of each season.

Likewise, there are no clinical features that distinguish infections of influenza B or C from influenza A. Myositis is more frequently, but not exclusively, associated with influenza B infections (Daley et al., 2000). Yamagata-like strains of influenza B may cause more invasive disease than Victoria-like strains (Chi et al., 2008). Influenza C infections are infrequently detected. Most reported cases of influenza C are in children and occur sporadically or as localized outbreaks. Mild URTIs are the most common manifestation, although in one large series of 170 symptomatic Japanese children, 11% were hospitalized with pneumonia, bronchitis, or bronchiolitis. Most admitted children were under 2 years of age (Matsuzaki et al., 2006). The paucity of influenza C reports, however, may be due to inability to recover the virus. Indeed, a majority of children worldwide acquire antibodies to influenza C early in life (Nishimura et al., 1987). The largest reported influenza C outbreak occurred across Japan in 2004, concurrent with a seasonal influenza A epidemic. During this time, 17% of specimens yielded influenza A and 2% contained influenza C. Half of all influenza C-positive specimens were detected solely by NAT (Matsuzaki et al., 2007).

Complications of influenza occur sporadically in otherwise healthy individuals but are most frequent in the elderly, children less than 2 years of age, immunocompromised persons, individuals with concurrent conditions such as heart, lung, or metabolic disease, and pregnant women, particularly those in the third trimester (Neuzil et al., 1998; Henrickson et al., 2004; Kim et al., 2007). Upper airway complications include otitis media and sinusitis (Chonmaitree et al., 2008). Primary viral pneumonia can occur at any age and is associated with significant mortality, particularly in recipients of HSCT and lung transplants (Garbino et al., 2004b; Khalifah et al., 2004; Kumar et al., 2005; Kim et al., 2007). Secondary bacterial pneumonia is a feared complication that often develops during serious influenza virus infections, particularly in the elderly and other high-risk patients (Cate, 1998; Hageman et al., 2006). The onset of productive cough with purulent sputum, new fever spikes, chest pain, and shortness of breath portend onset of bacterial superinfection. Common causes are *S. pneumoniae*, *S. aureus*, or *Haemophilus influenzae*. Mortality is high, especially in high-risk patients, even with prompt antibiotic treatment. This unique association between bacterial superinfection and influenza A may be due to viral NA-induced changes in the respiratory epithelium that permit increased bacterial adherence, decreased mucocilliary clearance, or impaired inflammatory cell function (Peltola et al., 2005; Avadhanula et al., 2006). Infections with common upper airway bacteria may have been responsible for most deaths during the devastating 1918 "Spanish flu" (Morens et al., 2008). Recently, clusters of fatal methicillin-resistant *S. aureus* infections secondary to seasonal influenza A have been reported in otherwise healthy U.S. children and adults, probably reflecting the increased colonization rate of the general population with this organism and its increased virulence (Bhat et al., 2005; Hageman et al., 2006). Mixed infections with influenza and other viruses are described, but it is unclear if the prognosis is worse than infection with influenza virus alone (Iwane et al., 2004).

Nonrespiratory complications of influenza are less frequent but can be equally devastating. Children, particularly of Japanese descent, are especially susceptible to secondary neurologic involvement (Morishima et al., 2002). Manifestations include encephalitis, acute disseminated encephalomyelitis, Guillain-Barré syndrome, transverse myelitis, Reye's syndrome (associated with aspirin use), postinfectious encephalitis, and acute necrotizing encephalopathy. Other complications include cardiomyopathy and myositis (including rhabdomyolysis). Morality rates are the highest among the elderly. For example, influenza-associated pulmonary and circulatory deaths in the United States during 1990 to 1999 per 100,000 persons were 98.3 among persons aged 65 years and older, compared to only 7.5 among persons aged 50 to 64 years and 0.4 to 0.6 among persons 40 years or younger. Individuals over 85 years of age were at highest risk. In contrast, fatality rates among children typically average about 0.4 per 100,000, or 92 deaths per year (Centers for Disease Control and Prevention, 2008). In 2003 to 2004, however, a troubling 153 deaths were reported in U.S. children secondary to influenza A infections (Louie et al., 2006). Over half of these fatalities were in children without underlying medical problems. Enhanced monitoring of influenza-associated mortality rates in U.S. children has now been implemented.

Influenza is the only respiratory virus illness for which antiviral treatment and vaccination are available. The most effective strategy for influenza control is annual vaccination. In the past, emphasis in the United States was placed on vaccination of the elderly due to their higher mortality following influenza virus infection than other age groups. Recent efforts include promoting universal vaccination of children to reduce their burden of disease and decrease their ability to spread the virus to others. The benefit of this approach to the community at large and the elderly in particular was recently demonstrated by vaccinating all Japanese schoolchildren (Reichert, 2002). Annual influenza vaccination in the United States is now recommended for individuals aged 6 months and older (Centers for Disease Control and Prevention, 2008).

Two types of vaccine are available, the trivalent inactivated influenza vaccine (TIV) and a similarly trivalent, live attenuated influenza virus vaccine (LAIV). Both vaccines contain two influenza A strains with the newest HA and NA surface antigens and a current type B strain. Vaccine strains are selected from candidates predicted to circulate in the upcoming winter. They are chosen annually by the WHO, based on analyses of strains circulating in late winter. Vaccine is then manufactured during the summer and distributed to providers by early autumn. Despite this temporal challenge, vaccines were well matched to circulating strains in 16 of the last 19 U.S. influenza seasons. Currently in the United States, vaccines are prepared from viruses grown in embryonated chicken eggs. For TIV, virus is harvested and disrupted, and the surface molecules are purified. Preparations contain standard amounts of HA and N molecules of an influenza A (H1N1), influenza A (H3N2), and influenza B strain. The vaccine is noninfectious and is often referred to as "subunit" or "split" virus vaccine. It is administered intramuscularly. TIV is currently approved in the United States for persons 6 months or older, including those with chronic medical conditions. In contrast, LAIV contains whole infectious virus. It is prepared by inserting the HA and N genes from each recommended strain into attenuated, master donor viruses

containing 6 internal genes. LAIV is administered intranasally, followed by transient replication of the virus in the upper respiratory tract. It is currently approved in the United States for healthy persons aged 2 to 49 years of age. Because it contains live virus, LAIV is not recommended for immunocompromised persons, the elderly, or persons with reactive airway disease (Centers for Disease Control and Prevention, 2008). Shedding of vaccine virus by otherwise healthy individuals is detectable in rapid antigen assays for about a week, so recent administration of LAIV should be considered when interpreting results of these tests (Ali et al., 2004).

The two vaccines elicit different immune responses. TIV evokes a higher systemic IgG antibody response. LAIV causes a better IgA mucosal antibody response, which may improve protection against primary infection in children and antigenically drifted strains, and durability of protection. If the vaccine strains are well matched to the circulating strains, TIV is 70 to 100% effective in preventing infection among healthy adults, and 30 to 60% effective in elderly individuals or young children (Centers for Disease Control and Prevention, 2008). Data comparing the efficacy of TIV to LAIV are still limited, although LAIV appears to be somewhat more effective in young children than adults (Ohmit et al., 2006; Belshe et al., 2007). Newer approaches to influenza vaccine are advanced. They include tissue culture-derived vaccine, more immunogenic vaccines made with concentrated antigen preparations or novel adjuvants, reverse genetic systems to accelerate TIV preparation, use of baculovirus-derived peptides, and DNA vaccines (Belshe, 2007).

Treatment is a useful adjunct in the prevention of influenza if implemented within 48 h of onset of symptoms. Currently in the United States, treatment is recommended for hospitalized patients with influenza, primary influenza pneumonia, or secondary bacterial pneumonia. It is also recommended for persons with influenza presenting to medical care who want to reduce the duration or severity of their illness or spread to others (Centers for Disease Control and Prevention, 2008). Laboratory confirmation of influenza is recommended prior to treatment but is not required. Appropriate use of chemoprophylaxis includes unvaccinated household contacts of high-risk individuals or asymptomatic high-risk individuals after exposure to a confirmed case.

Two classes of drugs, the amantadanes and the NA inhibitors, are currently licensed in the United States for treatment or prophylaxis of influenza. The amantadanes, amantadine and rimantidine, were the first anti-influenza medications developed. They are effective only against influenza A. Their mechanism of action is blockage of the virion M2 ion channel, thereby preventing virus uncoating (Wang et al., 1993). Adverse effects of amantadine involving the central nervous system have been observed, particularly in the elderly (Keyser et al., 2000). Recently, over 96% of circulating influenza A (H3N2) and 4% of influenza A (H1N1) isolates in the United States were found to contain a mutation conferring amantane resistance; higher values are detected in some other countries (Deyde et al., 2007). Therefore, amantadanes are not currently recommended for treatment of influenza A infections except when there is resistance to other antivirals (Centers for Disease Control and Prevention, 2008).

The competitive NA inhibitors, oseltamivir and zanamivir, are the current drugs of choice for influenza treatment and chemoprophylaxis. They are effective against influenza A and B, although lower efficacy against influenza B is reported for oseltamivir (Sugaya et al., 2007). Oseltamivir is administered orally. It is licensed in the United States for treatment or prophylaxis of children over a year of age and adults. Zanamivir is a dry powder that is inhaled. It is approved in the United States for treatment of patients over 7 years of age and prophylaxis for individuals 5 years of age and older. Both drugs are administered for 5 days, with dosages varying by the vacinee's age and medical condition (Centers for Disease Control and Prevention, 2008). Zanamivir is not recommended for individuals with underlying airway disease due to concerns for bronchospasm. Transient neurologic side effects of oseltamivir have been noted, particularly in Japan (Centers for Disease Control and Prevention, 2008). Treatment within 48 h of onset of symptoms (and especially within the first 12 h) with either drug shortens the duration of illness by about a day compared to placebo and may reduce complications as well (Cooper et al., 2003). Good-to-excellent efficacy for chemoprophylaxis is reported, especially in children.

Development of NA-resistant viruses and their transmission during treatment is infrequent in otherwise healthy individuals (Kiso et al., 2004). In contrast, severely immunocompromised patients may shed resistant influenza A and influenza B viruses for prolonged periods, even after cessation of treatment (Ison et al., 2006). Global resistance of influenza A and B to NA inhibitors is rising. For example, by the end of the 2007 to 2008 winter season in the United States, approximately 10% of influenza A (H1N1) viruses, but not influenza A (H3N2) or influenza B isolates, had acquired a mutation conferring oseltamivir resistance; zanamivir resistance was still rare. Higher levels of resistance are documented among influenza A isolates from the 2008 Southern Hemisphere winter season. Alternative approaches to treatment and chemoprophylaxis of influenza are therefore of great interest and are actively being sought. Compounds under investigation include conjugated sialidases, HA inhibitors, small interfering RNA (siRNA) molecules, polymerase, and protease inhibitors. Parenteral and topically applied, long-acting NA inhibitors are in clinical trials (Ong and Hayden, 2007). Testing for antiviral resistance is not currently available in clinical settings.

When decisions regarding treatment or infection controls are necessary, laboratory confirmation of influenza virus infection is desirable. Methods for virus detection include culture, serology, antigen detection, or NAT. A variety of upper respiratory tract specimens alone or in combination is typically used, although virus lower airway specimens can also be tested. Nasal or NP specimens have higher yield than throat swabs; pooled throat and nasal swabs can also be used (Weinberg et al., 2004). Test results are most reliable from specimens collected early (2 to 3 days) after onset or from young children because these samples presumably contain higher quantities of detectable virus (Landry et al., 2000).

Virus culture is the historical gold standard for influenza virus detection, but results are rarely available in time to guide treatment decisions. It remains useful to confirm results of other assays and to procure isolates for surveillance. Tube culture is typically performed using primary monkey kidney cells or continuous cell lines such as MDCK or LLC-MK2, which can display CPE in 2 to 5 days. CPE may also be inapparent, requiring hemadsorption of added guinea pig (influenza A and B) or chicken (influenza C) red blood cells to identify infected cells. Identification of isolates by clinical laboratories is usually performed by detection of antigen by IF and more recently by NAT. These newer methods correlate well with classic but labor-intensive hemagglutination inhibition methods and are increasingly used for subtyping as well (Atmar, 2007). Centrifugation-enhanced rapid culture using human or mixed cells in addition to the cell types

mentioned above can provide accelerated results (1 to 3 days) with 50 to 94% sensitivity compared to tube culture (Espy et al., 1986; Rabalais et al., 1992; LaSala et al., 2007). Serology has limited diagnostic usefulness, particularly in previously infected individuals, by requiring demonstration of a fourfold rise between acute- and convalescent-phase sera. It is most useful for surveillance or when respiratory tract samples cannot be obtained. Antibodies to influenza can be demonstrated by IA, complement fixation, neutralization, or inhibition of hemagglutination (Atmar, 2007). Serologic detection of subtype- or strain-specific responses is complicated by the fact that an anamnestic response to infection is highest to the original infecting virus, even when there have been subsequent infections with other strains. This phenomenon is called "doctrine of original antigenic sin."

Assays for influenza virus antigen are used by clinical laboratories as stand-alone tests or to screen specimens prior to further testing. Direct or indirect IF is popular with many full-service hospital laboratories. This method can detect and type influenza virus directly from patient samples in 2 to 4 h, with sensitivities of 60 to 95% depending on specimen type and patient population, and specificities of >95% compared to culture (Landry and Ferguson, 2000; Uyeki, 2003). Microtiter plate-based enzyme IAs (EIAs) have similar turnaround times and performance characteristics but are most applicable in reference laboratories for batch testing. The technical skill and equipment required for IF or EIA often precludes their performance around the clock or at the point of care. Therefore, rapid IAs that detect influenza virus antigen in 30 min or less are increasingly preferred by clinicians. Over 10 such kits are now available in the United States. A common format is a single-use immunochromatography cartridge. These IAs vary by many factors, including the type of specimen approved and ability to differentiate influenza A from B; several can be used in near-patient settings such as emergency rooms or clinics. Package inserts list sensitivities of 70 to 75% and specificities of 90 to 95%, although in practice, performance is usually lower. Predictive values also vary by the prevalence of influenza in the community, so these tests should be used only when influenza is widely circulating. For example, it has been suggested that virus prevalence in children should be at least 10% before influenza rapid antigen test results are reliable (Grijalva et al., 2007). When a clinical decision is important and rapid antigen test results are negative, specimens should be retested by a more sensitive and specific assay such as viral culture or a NAT (Centers for Disease Control and Prevention, 2008). A list of currently available rapid tests for influenza and clinical considerations for their use is available on the CDC website at http://www.cdc.gov/flu/professionals/diagnosis/index.htm.

Molecular diagnosis of influenza can now be accomplished with high sensitivity in less than a day by a number of conventional and real-time NATs (Erdman et al., 2003; Henrickson et al., 2004; Kuypers et al., 2006). Use of influenza NAT in the United States is currently limited to laboratories certified for high-complexity testing, although point-of-care approaches are in development. All diagnostic NATs discriminate influenza A from B. Multiplexed systems are available to further differentiate influenza A H1 from H3 and detect other respiratory viruses as well; two such assays are approved for diagnostic use in the United States (Legoff et al., 2008; Pabbaraju et al., 2008). NAT typically improves influenza virus detection by 10 to 60% compared to IF or culture. The largest increases are observed by testing specimens from adults or immunocompromised patients, late samples, or specimens with low virus titer such as sputum or tissue (van Elden et al., 2002; Simpson et al., 2003; Maricich et al., 2004; Weinberg et al., 2004; Kuypers et al., 2006). Influenza virus loads can be quantified, but such measurements are mostly investigational at this time (Boivin et al., 2003a). Molecular surveillance for evidence of antigenic drift and shift is now being performed in research and U.S. public health settings. In addition, microarrays are under development that can rapidly and accurately identify all human and animal H and N influenza A subtypes in isolates or in patient specimens and discriminate vaccine-derived influenza from wild virus (Freed et al., 2007; Mehlmann et al., 2007; Quan et al., 2007). Use of NAT should greatly streamline influenza diagnosis and surveillance.

AI

Pandemic strains of influenza A originate wholly or in part from AI viruses. Aquatic birds are the natural host and harbor AI viruses of all known subtypes. Infections in waterfowl are typically asymptomatic and confined to the intestinal tract. Virus is excreted into the water, with infections perpetuated by the fecal-oral route. Cross-species transmission is infrequent and is most often identified in Southeast Asia where wild birds come into close and frequent contact with domestic fowl and mammals (Fig. 2). The outcome of AI infections in terrestrial birds, particularly chickens and turkeys, is highly variable. AI strains of low pathogenicity (LPAI) induce asymptomatic infection or mild intestinal disease. Highly pathogenic AI (HPAI) strains, however, can involve the birds' respiratory tract, as in human influenza, inducing outbreaks with high mortality in affected flocks. Human infections follow such outbreaks but were rare until recently. From 1980 to 2004, only sporadic cases or small clusters were identified, mostly in association with HPAI A (H7N7), A (H7N3), or A (N9N2) subtypes. Conjunctivitis was the most characteristic clinical finding. Mild influenza-like illnesses, however, occurred in several children infected during

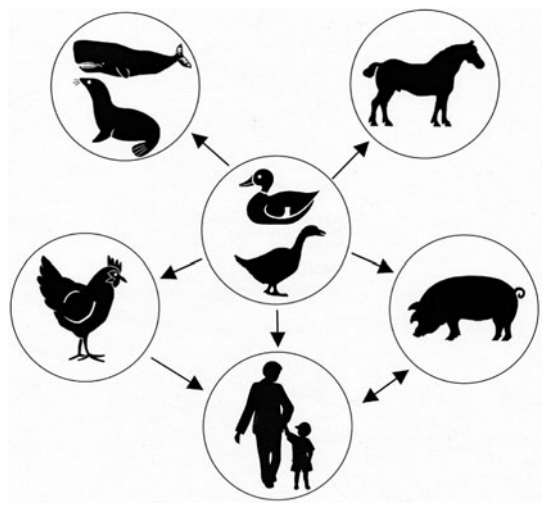

FIGURE 2 Transmission patterns of influenza A virus. The current hypothesis is that wild aquatic birds are the major reservoir of most types of influenza A, some of which can be transmitted to domestic birds and mammals. The double-headed arrow indicates that transmission of influenza A virus between pigs and humans has been demonstrated. Transmission of influenza A from birds to felines and dogs is not depicted. (Reprinted from Trampuz et al., 2004, with permission of the publisher.)

outbreaks of A (H9N2) infection in birds, and a veterinarian died of pneumonia after his involvement in a large poultry outbreak of A (H7N7) in The Netherlands (Peiris et al., 1999; van Kolfschooten, 2003).

In late 2003, an epizootic of unprecedented duration, geographic extent, and human involvement due to HPAI A (H5N1) influenza began. It started in 1996 as an outbreak among geese in southeastern China (Shortridge, 1998). The virus then reassorted multiple times with other AI strains and resurfaced in 1997, causing disease in poultry and other birds. The first reported transmission to humans was in Hong Kong with 18 cases, including 6 fatalities; the first death was in a 3-year-old child (Subbarao et al., 1998). Prompt slaughtering of 1.5 million domestic fowl and stringent infection control precautions in the poultry industry halted the outbreak. The virus continued to evolve and spread among birds, infecting three Hong Kong residents in 2003, one of whom died (Peiris et al., 2004). Concerns for global spread became acute when the virus unexpectedly killed thousands of wild birds in a large lake in western China in 2005 (Chen et al., 2005). Migratory birds have now spread the virus along natural flyways to more than 30 countries worldwide, including Russia and its neighbors, parts of Europe, the Middle East, and Africa. The impact of A (H5N1) on the global bird population was estimated in 2008 by the World Bank (http://siteresources.worldbank.org/EXTAVIANFLU/Resources/EvaluatingAHIeconomics_2008.pdf) to include the death or destruction of more than 500 million wild and domestic birds and worldwide losses of more than $10 billion to the poultry industry. The virus remains entrenched wherever humans and birds live in close proximity, although large-scale culling of birds in some countries appears to have eradicated the virus at least temporarily. Practices such as tending backyard flocks, shopping at live animal markets, and trade of exotic birds continue to increase the likelihood of virus transmission to humans and further evolution by mutation or reassortment with seasonal influenza into a pandemic strain. Indeed, an Indonesian teenager was recently found to be coinfected with A (H5N1) and the A (H3N2) strain circulating among humans. Meanwhile, the virus continues to evolve. Nine geographically distinct clades and several subclades of A (H5N1) are now identified, some of which have acquired the ability to infect other mammalian hosts such as tigers, domestic cats, and dogs (Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza A [H5N1] Virus [Writing Committee], 2008).

Despite the current high prevalence of A (H5N1) in birds, human infections remain relatively uncommon. As of September 2008, 387 human cases were confirmed, with a case fatality rate of 63%. The majority of cases and fatalities are reported from Indonesia and Vietnam. Current AI statistics are available at the WHO AI website at http://www.who.int/csr/disease/avian_influenza/country/en/. The median age of infection is 18 years, although individuals of all ages have been affected. Most were previously healthy. The case fatality rate is highest among 10- to 19-year-olds, with older adults underrepresented. Direct transmission from birds to humans is the predominant route of infection; handling of sick or dead poultry is the most common recognized risk factor. Most infections stem from poultry raised inside or outside the house. Asymptomatic infection is infrequent (Vong et al., 2006). Person-to-person transmission is still rare, with most reported clusters of cases linked to common-source exposures. Limited, nonsustained, human-human transmission, however, was likely in several cases during close, unprotected contact with secretions from severely ill individuals (Ungchusak et al., 2005; Writing Committee, 2008).

The incubation period of A (H5N1) in humans is less than a week. Patients typically present with a nonspecific, febrile illness accompanied by cough and shortness of breath. Chest radiographs demonstrate diffuse and patchy infiltrates; pleural effusions are rare. Nausea, vomiting, encephalopathy, and mucosal bleeding are sometimes reported. Watery diarrhea was present in many initial cases but has been rare since 2005. Mild febrile URTIs have occurred in some children recently, although prompt recognition and treatment may have reduced disease severity (Kandun et al., 2006). Laboratory abnormalities include significant lymphopenia and leucopenia, mild-to-moderate thrombocytopenia, and elevated transaminases. Fatalities usually occur 9 to 10 days after presentation and result from progressive viral pneumonia or acute respiratory distress syndrome (Rajagopal and Treanor, 2007). Limited autopsies have revealed diffuse alveolar damage with hyaline membranes, interstitial lymphoid and plasma cell infiltrates, bronchiolitis, and pulmonary congestion with hemorrhage, with evidence of virus in alveolar pneumocytes and macrophages (Ng et al., 2006). High concentrations of virus are also found in throat or trachea, but relatively little virus is present in the nose. This distribution differs significantly from seasonal influenza and may partially explain the infrequency of person-to-person transmission.

Shedding from the respiratory tract can persist for 3 weeks and occasionally longer, probably related to an ineffective immune response or virus evasion. Dissemination is suggested, particularly in fatal cases, by the presence of virus in blood, cerebral spinal fluid (CSF), viscera, and stool. Higher levels of cytokines occur in patients infected with H5N1 than with seasonal influenza strains (Chan et al., 2005). The highest viral loads are in fatal cases (de Jong et al., 2006). These findings suggest that deaths probably reflect lung and other tissue damage due to the combined effects of unrestrained viral replication and the host inflammatory response.

The low transmissibility of A (H5N1) from birds to humans suggests a strong natural barrier to cross-species infection. One such restriction may act during virus attachment. AI viruses preferentially bind to cellular receptors of birds which contain α2,3 sialic acid-linked glycans. Since cells with α2,6 linkages predominate in the human upper airway, the paucity of receptors preferred by AI probably limits infection (Connor et al., 1994). Interestingly, swine possess both linkages, which may explain their proposed role as a source of reassortants between human and AI strains (Ito et al., 1998). The severity of human A (H5N1) infections may also relate to cellular receptor distribution. Unlike the α2,6 linkages in the upper respiratory tract, human bronchioles and alveoli are rich in α2,3 linkages. Preferential attachment of A (H5N1) virus to favored receptors in the human lower airway could induce especially severe disease due to compromise of gas exchange following local virus replication and a vigorous cytokine response. Of considerable concern is that A (H5N1) strains have been isolated from humans that can infect human cells with either receptor type (Yamada et al., 2006). These novel strains do not appear to be more transmissible, suggesting that additional viral changes are needed to promote human-to-human spread. One such change may be a mutation that all HPAI viruses have acquired—a polybasic amino acid sequence in the HA molecule conferring increased cleavability, permitting virus attachment to a wider range of tissues (Govorkova et al., 2005). Changes in internal genes such as M, NP, and PB may also be required for efficient spread. Indeed, a mutation in

PB2 has recently been detected that that permits AI replication in the upper airway of mammals and could improve efficiency of person-to-person transmission (Hatta et al., 2007). Recently, novel A (H7N2), A (H7N3), and A (H9N2) AI strains have emerged with A (H5N1)-like properties, suggesting that other influenza subtypes might have pandemic potential (Belser et al., 2008; Wan et al., 2008).

Initial diagnosis of human A (H5N1) disease is best established by detection of viral RNA using NAT, which can be safely performed under biosafety level 2 (BSL-2) conditions. Conserved genes such as M or NP should be targeted, using updated primers and probes for currently circulating strains. Microarrays are under development to rapidly identify PCR products from all influenza H and N influenza A subtypes and discriminate among different A (H5N1) clades (Mehlmann et al., 2007; Quan et al., 2007). Diagnostic yield is best with throat swabs and lower airway specimens due to higher viral loads in deeper structures (de Jong et al., 2006). Collection of nasal swabs is also recommended in case the illness is caused by seasonal influenza strains. Recovery of virus from sources such as blood, feces, or CSF is lower than from the respiratory tract. Although A (H5N1) can be grown on the same cell lines as seasonal influenza, culture should only be attempted by laboratories meeting BSL-3 or above requirements. Most commercially available rapid influenza A antigen tests have similar analytical sensitivities for AI and seasonal influenza subtypes but cannot differentiate seasonal influenza virus from AI (Chan et al., 2002; Chan et al., 2007). Seroconversion to A (H5N1) infection is usually demonstrable 2 to 3 weeks after infection. Detection of antibody is therefore useful mostly for epidemiologic purposes and to establish diagnoses retrospectively. A fourfold rise in antibodies or a single high titer suggests infection. Nonpathogenic H5N1 viruses have recently been constructed by reverse genetics and other means that may permit serologic testing in BSL-2 level facilities. See the WHO AI website referenced above for current diagnostic approaches to circulating AI strains.

Many A (H5N1) strains are resistant to the amantadanes, so oseltamivir is the current antiviral of choice (Writing Committee, 2008). Resistance of A (H5N1) to oseltamivir is currently rare. Limited clinical trials suggest that early treatment is beneficial and improves survival, although mortality remains high (Cooper et al., 2003). Higher levels and more prolonged administration are required to suppress replication of some strains in vitro and in animal models, so a twofold-higher dosage for a prolonged course of 10 days is proposed. Resistance has been detected in some treated patients, so combination treatment with amantadine is suggested in countries where A (H5N1) viruses are likely to be susceptible (Govorkova et al., 2004; Writing Committee, 2008). The value of inhaled zanamivir has not been studied, due partially to concerns regarding suboptimal delivery to the lungs of ill patients. Intravenous zanamivir and a third drug, peramivir, are under development for use in these situations. Corticosteroids may cause adverse reactions but may be considered for shock or adrenal insufficiency. The benefit of immunomodulators remains unproven. Some mathematical models predict that combined use of targeted chemoprophylaxis with oseltamivir and social distancing might extinguish or delay a large A (H5N1) outbreak with human-human transmission in rural Asia (Monto et al., 2006). Chemoprophylaxis may also be useful for people at high risk for transmission, such as poultry workers, family members of a suspected or confirmed case, or healthcare workers. Therefore, oseltamivir has been stockpiled by the WHO and many countries for distribution as needed. Current recommendations for treatment and chemoprophylaxis are available on the WHO AI website.

Development of a safe and effective vaccine is considered the best and most cost-effective way of limiting an influenza pandemic. Such efforts for A (H5N1) were initially hampered by the weak immunogenicity of the H5 antigen in humans requiring high doses for an initial antibody response and the rapid mutation of circulating strains requiring vaccines prepared from multiple lineages or capable of inducing cross-clade protection. In addition, antibody levels required for protection of humans against A (H5N1) illness remain unclear. Safe and immunogenic inactivated A (H5N1) vaccines have now been developed (Treanor et al., 2006). Systems to generate seed viruses with increased antigen yield in chicken eggs and egg-independent vaccine systems will soon be available (Hoelscher et al., 2006; Ehrlich et al., 2008). Dose-sparing strategies such as use of oil-in-water adjuvants and prime-boosting immunization schedules now exist which reduce the amount of H5 antigen required for an initial antibody response, induce cross-reactive antibodies against drifted strains, and can extend the antibody response in primed individuals up to 8 years (Poland and Sambhara, 2008). Interestingly, serologic surveys indicate that some older adults appear to have some level of pre-existing antibodies against A (H5N1), although the mechanism resulting in these antibodies is unclear. The continued concern about a pandemic due to A (H5N1) has prompted Japan to begin vaccination of high-risk individuals with an inactivated A (H5N1) preparation in 2009. The United States has approved a similar vaccine for first-responders, laboratory workers, and others in other high-risk professions, and stockpiling of a split-virus vaccine was recently approved in Europe. Efforts to improve pandemic influenza vaccines continue, however, and include evaluation of live virus vaccines, conserved antigen vaccines, and development of more-effective adjuvants. This significant progress suggests that vaccination may become part of the global strategy to limit pandemic influenza.

RSV

Human RSV is the most important etiological agent of acute lower respiratory tract illness in infants and young children worldwide. The virus was first isolated in 1956 from a symptomatic laboratory chimpanzee and later named for its characteristic CPE of syncytia formation in monolayer culture. Subsequent serologic and epidemiology studies demonstrated RSV as the major viral cause of bronchiolitis and pneumonia in children less than 5 years of age (Brandt et al., 1973; Collins et al., 2001). In the United States each year, RSV is associated with approximately 60,000 to 100,000 hospitalizations, over 100 deaths, and more than $652 million in costs for medically attended visits in this age group (Leader and Kohlhase, 2003; Glezen, 2004; Paramore et al., 2004). The virus has also been identified as a cause of serious LRTI in other populations, including the elderly and immunocompromised patients (Hall et al., 2001; Falsey, 2005; Kim et al., 2007).

RSV is classified within the *Paramyxoviridae* family of nonsegmented, negative-strand, enveloped RNA viruses (Table 1). It is further subcategorized within the *Pneumovirinae* subfamily by lacking the HA or HA-NA (HN) attachment molecules that the *Paramyxoviridae* subfamily contains (Collins et al., 2001). Virions are irregularly spherical and 150 to 300 nm in diameter, with filamentous forms also observed. They possess an RNA genome of approximately

15 kb encoding at least 10 proteins. The N surrounds the viral RNA and is the major component of the nucleocapsid. Within the nucleocapsid is also the RNA-dependent RNA polymerase complex consisting of the large (L) protein, the phosopho (P) protein, and transcription/replication factors M2-1 and M2-2. The matrix protein (M) surrounds the nucleocapsid and links it to the three surface glycoproteins, glycoprotein (G), fusion (F) protein, and the short hydrophobic (SH) protein. The viral glycoproteins are organized into short spikes which project from the viral envelope. There are also 2 nonstructural proteins designated NS1 and NS2. The G protein and, perhaps, the F protein mediate attachment to host cells. The F protein is responsible for membrane fusion, which is important to virus entry into host cells and spread of infection by syncytia formation involving infected and uninfected cells. It may also be important in attachment to cellular receptors. The F and G proteins are the primary targets of the immune response and the most promising antigens for subunit vaccines (Power, 2008).

There are two RSV subgroups, A and B, based on antigenic and sequence analysis. The most extensive difference between them lies in the G protein. The G proteins of each subgroup share only 53% amino acid homology and 5% antigenic relatedness. In contrast, the F proteins have 89% amino acid sequence homology and are 53% antigenically related (Sullender, 2000). Subgroup differentiation has epidemiologic and some clinical relevance. For example, initial infection with a subgroup A virus provides some degree of protection against reinfection by subgroup A viruses but not with subgroup B (Mufson et al., 1987). This one-way pattern probably contributes to the predominance of subgroup A outbreaks. Viruses from each subgroup may cocirculate or one may predominate in a community in an epidemic year; viruses at nearby locales can be the same or quite different (Hall et al., 1990). Antigenic and genetic variability also exist within each subgroup (Storch et al., 1991; Peret et al., 1998). This diversity probably arises from local immunologic pressure, resulting in selection of new strains that spread in successive outbreaks. Indeed, even though a single subtype may persist for more than 1 year, the viruses usually have genotypic differences, suggesting that the virus is constantly evolving. Infections with subgroup A viruses may be more severe than subgroup B infections, although this association is not uniformly observed (Walsh et al., 1997). Viruses of subgroup A replicate to the highest titers in culture and the human respiratory tract. Determinants of disease severity, however, are likely to be complex and involve viral load as well as host genetics and other virulence factors (Crowe and Williams, 2003; Devincenzo, 2005). Currently, subtype identification and viral load have no importance in clinical decision making.

Most RSV infections are acquired by contact with contaminated secretions in droplets or on fomites; aerosol transmission is infrequent. Although RSV is relatively labile compared to other respiratory viruses, it can persist on surfaces for up to 6 h and on hands for about 30 min (Hall et al., 1980; Blydt-Hansen et al., 1999). Transmission is highly efficient. During an outbreak, over 50% of babies born in the previous spring and summer are infected. Almost all children have been infected by 2.5 to 3 years of age due to exposure during successive outbreaks. Transmission within households is common. Spread of virus is facilitated by shedding from infants for up to 4 weeks and from immunocompromised persons for months after infection; older children and adults shed only for a few days. Factors increasing attack rate include crowding and exposure to tobacco smoke. Following infection, the virus replicates in the cytoplasm of superficial NP cells. URTI symptoms of cough, sneezing, and rhinorrhea usually begin 4 to 8 days after infection; symptoms may be minimal in very young or preterm infants, but otherwise, few infections are asymptomatic. Other indicators are lethargy, irritability, poor feeding, or apneic episodes. Otitis media can occur in up to half of all cases and is an important driver of medical costs (Paramore et al., 2004; Chonmaitree et al., 2008). Most children recover uneventfully after 8 to 15 days.

Lower airway involvement requiring hospitalization occurs in 25 to 40% of pediatric cases. Spread of virus to the lower respiratory tract probably involves aspiration of secretions accumulating from virus-induced impairment of the ciliary beat; lateral spread of virus is inefficient (Devincenzo, 2005). Symptoms usually begin quickly, 1 to 3 days after infection, and manifest as bronchiolitis or pneumonia. Most affected children are previously healthy. Factors predisposing to LRTI requiring hospital admission include male sex, birth during the first half of the season, and low weight. Altitude above 2,500 meters, lack of breastfeeding, and tobacco smoke exposure are modest predictors (Simoes, 2003; Devincenzo, 2005; Choudhuri et al., 2006). Most patients improve with supportive care and are discharged in fewer than 5 days. Increased risk of severe disease and mortality is observed with premature birth, immunosuppressive conditions, or underlying cardiac, pulmonary, or neuromuscular disease (Henrickson et al., 2004; Purcell and Fergie, 2004).

The pathogenesis of severe RSV-associated LRTIs in the pediatric age group is still unclear. Concurrent invasive bacterial coinfections are infrequently encountered unless patients are immunocompromised or intubated (Purcell and Fergie, 2002; Jartti et al., 2004; Kneyber et al., 2005; Thorburn et al., 2006). It was once believed that RSV replication triggers an inappropriately excessive immune response. Recent evidence, however, favors rapid and profound RSV replication leading to massive apoptotic sloughing of respiratory cells and airway obstruction (Bennett et al., 2007; Johnson et al., 2007; Welliver et al., 2007). Indeed, the adaptive immune response of severely affected infants may be inadequate compared to the response induced by most other viruses. Factors that may correlate with lack of responsiveness may include recently identified host genetic variations in surfactant proteins A and D, Toll-like receptors, and cytokine production (Moore and Peebles, 2006).

Extrapulmonary manifestations may also occur during RSV infection, but whether they represent direct or indirect effects of the virus is unclear. In several studies, viral RNA was detected in feces, blood, and CSF of RSV-infected children by NAT (Rohwedder et al., 1998; Zlateva and Van Ranst, 2004; von Linstow et al., 2006). Infectious virus was also isolated from the liver, kidney, and myocardium of several fatal cases of RSV in severely immunocompromised infants and adults but was probably not the cause of death (Collins et al., 2001). It has been proposed that extrapulmonary effects may contribute to the inexplicable clinical instabilities seen in some RSV-infected patients and, perhaps, to sudden infant death syndrome. Additional studies are required to clarify the frequency and consequences of RSV spread beyond the respiratory tract (Eisenhut, 2006; Fernandez-Rodriguez et al., 2006).

Following recovery from RSV LRTI, a subset of pediatric patients has persistent morbidity. Prematurely born infants, in particular, may have residual abnormal lung function at follow-up (Broughton et al., 2007). Other children will develop recurrent wheezing or asthma which usually decreases

in later life; some children exhibit these symptoms with reinfection in subsequent years. It remains unclear whether RSV directly triggers reactive airway disease or whether infection with RSV or other respiratory viruses reveal pre-existing host abnormalities (Gern, 2004). Indeed, rhinoviruses were detected more frequently than RSV in a recent, controlled study of study of asthma exacerbations in children (Khetsuriani et al., 2007).

For reasons that are not understood, immunity to RSV infections is incomplete and short-lived. During initial infection, about half of all infected infants will produce specific IgM, IgG, and IgA in respiratory secretions. These antibodies, however, are low in titer and avidity due to immunological immaturity and/or suppressive effects of maternal antibodies (Crowe and Williams, 2003). They also wane rapidly and may completely disappear by a year of life, especially if infection occurred in early infancy. Repeated RSV infections are common throughout life, with reinfection rates as high as 20 to 40% occurring among older children during outbreaks. Hospitalization without underlying chronic disease, however, is infrequent (Tsolia et al., 2004). Longer-lasting antibodies begin to appear after such reinfections and probably contribute to the milder symptoms observed beyond early childhood (Hall et al., 1991). The importance of cell-mediated immunity in recovery from RSV disease is demonstrated by the severe consequences of RSV infection in immunocompromised patients. Cytotoxic T cells and an array of cyokines do appear in the blood and secretions of infants or adults during RSV infection, but they differ little from those induced by other viruses (Bennett et al., 2007). Further studies are required to understand the weak and impermanent nature of the RSV immune response (Chung et al., 2007; Power, 2008).

Reinfections in later life, however, are not always benign. In a prospective study of otherwise healthy working adults, RSV infections were observed in 7% of subjects. These illnesses were associated with more prolonged symptoms than influenza and with appreciable costs for medical visits and absence from work. Progression to LRTI was observed in 26% of cases (Hall et al., 2001). Morbidity and mortality are especially high in the elderly and high-risk adults. In 608 elderly and 540 other high-risk adults, RSV accounted for 10.6% of hospitalizations for COPD, 7.2% of hospitalizations for asthma, and 5.4% of hospitalizations for congestive heart failure. Mortality was 8% (Falsey, 2005). Indeed, RSV may be associated with up to 15,000 U.S. deaths annually in persons over the age of 65 (Thompson et al., 2003). Most of these infections are difficult to recognize clinically (Walsh et al., 2007).

RSV is also an important cause of serious LRTIs in immunocompromised patients (Mendoza Sanchez et al., 2006; Kim et al., 2007; Khanna et al., 2008). Recipients of HSCT are at the highest risk, especially in the first 100 days after transplantation and in patients who are older or do not engraft. Mortality of more than 50%, mostly due to viral pneumonia, was reported in early studies but is now lower due to improved management. The serious nature of RSV infections in this population, however, has prompted some centers to recommend postponing HSCT if RSV is diagnosed pretransplantation (Peck et al., 2004). The consequence of RSV infection in lung transplant recipients has been significant in some studies but not others (Milstone et al., 2006; Kim et al., 2007). Shedding of virus by immunocompromised patients can be prolonged, leading to progressive pulmonary damage and providing virus to infect others (Garbino et al., 2004a; Khalifah et al., 2004; Kim et al., 2007). Individuals with RSV-associated illnesses not serious enough to be homebound, particularly among family members and medical personnel, can introduce nosocomial infections to facilities caring for such patients.

Treatment of serious RSV infection is largely supportive at present. Corticosteroids or bronchodilators are generally ineffective (Corneli et al., 2007). The synthetic nucleoside ribavirin has antiviral activity against RSV in vitro and had been used in aerosol form to treat severe LRTIs in pediatric patients. This application has been largely discontinued due to questions of ribavirin efficacy, high cost, and difficulties with drug delivery. The drug does show some benefit, however, and continues to be used in special circumstances (Ventre and Randolph, 2007). For example, treatment of RSV in HSCT patients with aerosolized or intravenous ribavirin, often in combination with passively administered immunoglobulin, can reduce mortality, especially if initiated before respiratory failure (Kim et al., 2007). In one recent study of 18 lung transplant recipients, intravenous ribavirin and steroids were safe and effective against new RSV infections (Glanville et al., 2005).

Prevention of RSV infection has focused on active and passive immunization. Active immunization has been largely unsuccessful. Despite considerable research, there is currently no safe and effective vaccine against RSV. One experimental, formalin-inactivated, whole-virus vaccine actually primed young infants to develop enhanced RSV disease upon natural infection, resulting in hospitalization of 80% of vaccinees with subsequent RSV infection. Two vaccinated children died (Kim et al., 1969). Other hurdles to vaccine development include a requirement for efficacy in young infants in the presence of maternal antibody, a generally poor immune response of infants to natural RSV infection, and a need to protect against multiple strains of RSV subgroups A and B. Efficacy in the elderly as well as children would be desirable. Both subunit and live virus vaccine candidates are under investigation, but until mechanisms of RSV pathogenesis and host responses to infection are better understood, it is unlikely that a safe and effective vaccine will be available soon (Devincenzo, 2005; Power, 2008). Promising new compounds now in clinical trials for prevention or treatment include siRNA molecules that reduce RSV RNA replication in a sequence-specific manner (DeVincenzo et al., 2008).

By contrast, passively administered antibody has been quite effective at reducing RSV disease severity in high-risk infants and children and may benefit high-risk immunocompromised patients as well. A preparation of intravenous immunoglobulin with high titers of antibody to RSV (RSV-Ig) was introduced in 1996 and offered significant protection to high-risk children. High volumes were required for protection, and the preparation is no longer available. A year later, palivizumab was licensed to reduce RSV LRTI in selected infants and children with chronic lung disease of prematurity (also known as bronchopulmonary dysplasia), preterm birth, or congenital heart disease. Palivizumab (Synagis, Gaithersburg, MD) is a humanized mouse monoclonal antibody that neutralizes RSV by binding to the F protein with 50-fold-greater potency than RSV-Ig (Johnson et al., 1997). It is administered monthly by intramuscular injection before and during the RSV season and is expensive, so timing of administration should be coordinated with knowledge of local RSV circulation patterns whenever possible (DeVincenzo, 2008). A more-potent formulation of palivizumab antibody (motavizumab) with enhanced F protein binding and another preparation with an extended serum half-life (Numax-YTE) have been created and are

under evaluation (Wu et al., 2008). To date, palivizumab-resistant mutants have not been detected among clinical isolates, although escape mutants can be generated in vitro and in experimental animals (Devincenzo et al., 2004; Zhao et al., 2006).

Presumptive diagnosis of RSV in infants with bronchiolitis can be made during a community outbreak if HMPV is not circulating. Laboratory-based detection is required for accurate diagnosis, especially in other clinical settings and in older age groups (Wilkesmann et al., 2006). Virus culture, IAs, and molecular assays are most appropriate for this purpose. Serology is usually reserved for epidemiologic studies as it is insensitive and requires a convalescent-phase serum. Culture is the historical gold standard. The CPE of RSV develops in tube culture within 5 to 8 days on average and includes the formation of syncytia. Heteroploid cells such as A549 are currently preferred for tube culture by many laboratories over the previous standard of Hep-2 cells. Confirmation of positive cultures by another method such as IF, IA, or NAT should be performed, since other respiratory viruses can induce syncytia on occasion. Sensitivity of culture compared to other techniques ranges from 57 to 90%, with variations due to virus subtype, laboratory expertise, quality of the cells and specimen, and stage of infection. Tube culture is most successful in infants because virus is shed at higher titers and for longer periods than in adults (Falsey and Walsh, 2000). Centrifugation-enhanced culture is a useful alternative due to higher sensitivity and decreased time to detection (Rabalais et al., 1992; LaSala et al., 2007).

Rapid detection of RSV is useful to limit nosocomial spread and implement timely treatment in severe cases. Other advantages can include reductions in antibiotic use, ordering of fewer ancillary laboratory tests, and shorter lengths of stay (Woo et al., 1997; Barenfanger et al., 2000). Antigen detection is a widely used approach. Many kits for RSV antigen detection by IA or IF are commercially available. The IAs and newer flowthrough chromatographic formats, in particular, are rapid and easiest to perform and interpret, although performance characteristics are typically lower than for IF or culture (Ohm-Smith et al., 2004). Sensitivities of 59 to 97% and specificities of 75 to 100% compared to culture are reported using IA and upper airway specimens from pediatric patients (Henrickson and Hall, 2007). Detection rates are unacceptably low with upper respiratory tract specimens from immunocompromised patients and adults but can be improved by testing of lower airway samples (Englund et al., 1996; Falsey and Walsh, 2000; Kim et al., 2007). Specificities suffer when prevalence of the virus is low. When correctly performed, IF has higher sensitivity and specificity than IA, can be used with upper and lower tract specimens from children and many adults, and can discriminate RSV from other respiratory viruses when pooled monoclonal antibodies are used (Landry and Ferguson, 2000).

Detection of RSV RNA by NAT may become the new gold standard due to higher sensitivity than viral culture, IA, or IF (Henrickson and Hall, 2007; Casiano-Colon et al., 2003). Many good NAT assays are described in the literature, and a variety of chemistries have been used (Tang and Crowe, 2007). Several kits for RSV RNA detection by NAT are now commercially available and approved in the United States for use by high-complexity laboratories. Results can be provided in less than a day, RSV subtypes can be discriminated, and other respiratory viruses can be detected simultaneously (Legoff et al., 2008; Pabbaraju et al., 2008). In one recent study, use of NAT enhanced the recovery of RSV 2.6-fold compared to culture, a greater increase than was observed for influenza or parainfluenza virus types 1, 2, or 3. Molecular assays are also preferred to detect the low RSV titers shed by immunocompromised patients and adults.

HMPV

HMPV ranks as one of the most important causes of bronchiolitis in young children. It was identified in 2001 using a newly developed molecular pathogen discovery technique among unusual paramyxovirus-like isolates obtained over a 20-year period from young Dutch children with acute respiratory illnesses (van den Hoogen et al., 2001). The name reflects its classification as the first human member virus of the pre-existing *Metapneumovirus* genus which heretofore encompassed only avian viruses. Subsequent studies demonstrated that HMPV has been circulating in humans for at least 50 years and did not recently originate from birds. It is also ubiquitous, with virtually all children worldwide infected by 5 to 10 years of age (van den Hoogen et al., 2001; Ebihara et al., 2003; Wolf et al., 2003).

Like RSV, HMPV is a member of the *Paramyoxoviridae* family and the *Pneumovirinae* subfamily, but it belongs to the separate *Metapneumovirus* genus (Table 1). Its closest relative is an avian pneumovirus known as turkey rhinotracheitis virus. HMPV virions are enveloped and pleomorphic, ranging from spherical to filamentous in shape, with a diameter of about 200 nm. They encase a single-stranded, nonsegmented RNA genome of about 13 kb. There are 8 genes encoding at least 9 putative proteins. Like RSV, HMPV has an N protein, an L protein polymerase subunit, a P protein, transcription/replication factor proteins M2-1 and M2-2, and an M protein. Within the envelope are the attachment G protein, SH glycoprotein, and F glycoprotein. As with RSV, the G protein is the most variable among isolates, although it is much smaller than its RSV counterpart. This variation may aid immune evasion. The F protein is highly conserved among isolates and is similar in RSV and HMPV (van den Hoogen et al., 2002). It is also the immunodominant protein (Biacchesi et al., 2003). The major differences between HMPV and RSV are gene order and the absence of NS1 and NS2 genes in HMPV. Since NS proteins of RSV may have anti-interferon activity, their absence may contribute to the somewhat lesser pathogenicity of HMPV (Bossert and Conzelmann, 2002).

There are 2 major HMPV genetic subgroups (A and B) and at least 4 lineages (A1, A2, B1, and B2) (van den Hoogen et al., 2004). Homology between the subgroups is 80% at the RNA level and 90% at the protein level, with G and SH genes providing most of the variability (Biacchesi et al., 2003). Whether the two HMPV genogroups represent different serogroups as for RSV is controversial (Skiadopoulos et al., 2004; van den Hoogen et al., 2004). It has been suggested that genogroup A may cause more severe disease than genogroup B, but one study found no change in severity when a genogroup changed (Agapov et al., 2006; Vicente et al., 2006). Circulation of HMPV is community based, as with RSV, with predominant genogroups and lineages varying by location and annually (Peret et al., 2000). These patterns were further described in an extensive analysis of specimens collected from children over a 20-year period at a single location. All 4 genetic lineages were detected during the study period, but the amount of virus circulating varied considerably from year to year. Cocirculation of genogroups was observed. Reinfections were common and occurred with homologous and heterologous lineages. Asymptomatic infections were infrequent (Williams et al., 2006).

HMPV causes a broad spectrum of upper and lower airway illnesses that are clinically indistinguishable from the illnesses induced by RSV. Most initial infections occur during early childhood but somewhat later than with RSV (Wolf et al., 2006). The median age of infection was 20 months in one large U.S. study (Williams et al., 2006). Upper airway involvement is frequent and manifests primarily as the common cold. Rhinitis, coryza, cough, and fever are the most common clinical features. Overall, 5 to 15% of all URTIs in infants and young children appear to be due to HMPV, a proportion which is slightly lower than that induced by other common respiratory viruses. Otitis media can complicate HMPV URTIs in a third to half of all cases (Williams et al., 2006).

Lower airway involvement with HMPV is frequent, particularly in the first 6 to 12 months of life, suggesting that young age is a risk factor for more severe disease (Williams et al., 2004). Although estimates vary by location and year, the incidence of HMPV LRTIs appears to be about 5 to 15% (Kahn, 2006). Many cases require hospitalization. Overall, the virus ranks as the second or third most common viral cause of lower airway disease in admitted pediatric patients. Representative clinical features in one large study of HMPV LRTIs were bronchiolitis in 59% of cases, croup in 18%, and pneumonia in 8%. Cough, coryza, fever, wheezing, and dyspnea were frequent signs and symptoms, with diarrhea and vomiting also reported (Williams et al., 2004). Atelectasis, pneumonia, and exacerbations of asthma may be more frequently associated with HMPV LRTIs than with comparable RSV illnesses (Wolf et al., 2006). Some HMPV LRTIs require intensive care and mechanical ventilation. Extracorporeal membrane oxygenation was necessary in one reported case (Ulloa-Gutierrez et al., 2004). As with other respiratory viruses, risk factors for a severe course include a history of premature birth, underlying cardiopulmonary disease, or an immune system abnormality (Kahn, 2006). As a group, HMPV LRTIs tend to be similar or somewhat milder in severity than those of RSV (Boivin et al., 2003b; Wolf et al., 2006).

Some HMPV infections may cross the blood-brain barrier, although such occurrences are probably rare. Indeed, other members of the *Paramyxoviridae* family such as measles virus and Nipah virus are known to spread from the respiratory tract to the brain. Fatal encephalitis occurred in a 14-month-old child with HMPV URTI. Viral RNA was detected in brain and lung tissue samples (Schildgen et al., 2005). One case of encephalitis was identified among 29 Japanese children with HMPV and respiratory symptoms, although the CSF of this patient was not tested (Kaida et al., 2006). Finally, four cases of HMPV-associated encephalitis were identified in a multiyear study of etiologic agents of encephalitis in U.S. patients, although the specimen types tested and the number comprising the HMPV group were not given (Glaser et al., 2006). Further studies are needed to establish if the central nervous system effects of HMPV involve direct replication of the virus in the brain or an indirect effect, such as fever.

HMPV infections in immunocompromised patients vary in incidence and severity. Recipients of HSCT and lung transplants seem to be at highest risk of serious outcomes. Interestingly, HMPV was the predominant respiratory virus detected in two recent studies of symptomatic lung transplant recipients. Several deaths were associated with HMPV infection, and high viral loads in lower airway specimens were associated with significant lung cytopathology (Larcher et al., 2005; Gerna et al., 2006c; Dare et al., 2007). The incidence of HMPV-associated LRTIs in HSCT recipients is 3 to 4%, with progressive pneumonia and occasional fatalities reported (Englund et al., 2006). Many HSCT patients can also have persistent, symptomless HMPV infections (Debiaggi et al., 2006). Individuals with hematologic malignancies and severe combined immunodeficiency disease are also at risk (Kim et al., 2007; Williams et al., 2005; Abed and Boivin, 2008). HMPV was recently linked to higher rates of hospitalization and death in HIV-infected African children than in children not infected with HIV. Some of the severe and fatal infections in these patients involved bacterial coinfection, especially with *S. pneumoniae* (Madhi et al., 2007). Comparable studies in other countries are not yet available.

The potential for coinfections involving HMPV and other respiratory viruses, especially RSV, is high given their overlap in seasonality. Indeed, two European studies identified dual RSV and HMPV infections in over 60% of children with bronchiolitis who required intensive care, suggesting that coinfections may augment the disease process (Greensill et al., 2003; Konig et al., 2004). Caution is warranted, however, because the viral RNA detected could result from a recent prior infection. Other studies report either solo HMPV infections or coinfection rates of less than 10% without significant, additive clinical consequence (Jennings et al., 2004; Mullins et al., 2004; Richard et al., 2008). One notable coinfection is that of HMPV and the SARS-CoV. Before the CoV etiology of SARS was established, HMPV was recovered from some SARS cases, some of which had especially severe clinical courses (Chan et al., 2003). This association was brief and demonstrated only at some locales. No synergy between the two viruses was observed in the macaque model of SARS (Fouchier et al., 2003).

Repeated infections with HMPV, as with other respiratory viruses, are frequent in older children and adults (Falsey et al., 2003; Williams et al., 2004). Using NAT and serology, 1 to 21% of adults appear to be reinfected with HMPV each year (Falsey et al., 2003). Approximately two-thirds of these infections are symptomatic. Clinical findings include mild URTIs, bronchitis, pneumonia, an influenza-like illness, and a mononucleosis picture (Tsolia et al., 2004; van Gageldonk-Lafeber et al., 2005; Li et al., 2008). In some years, the number of affected adults may be quite high and result in a significant number of admissions, particularly among high-risk individuals. For example, one U.S. study of HMPV in adults reported infection rates of 1.5% to 7% over a 2-year period; in the second year, 11% of all hospitalized adults had evidence of HMPV infection. Most hospitalized subjects were elderly or had underlying cardiac or pulmonary problems (Falsey et al., 2003). As with RSV, HMPV infections in the frail elderly can cause significant morbidity and mortality. Dyspnea is a frequent clinical finding in elderly adults compared to younger individuals. One HMPV outbreak among elderly residents in a long-term care facility had a mortality rate of 50% (Boivin et al., 2007). Another outbreak occurred unexpectedly during the summertime (Louie et al., 2007). HMPV has been reported in exacerbations of asthma, congestive heart disease, and COPD, although controlled studies are still lacking (Williams et al., 2004; Beckham et al., 2005; Falsey, 2005; Khetsuriani et al., 2007).

Formal transmission studies of HMPV are not yet reported, but the virus probably is spread by large-particle secretions or fomites with subsequent infection of respiratory mucosal cells, as is RSV. The incubation period is estimated at 4 to 6 days based on a single case (Ebihara et al., 2004). Few histopathologic assessments are available, but one report of lung tissue from 5 subjects with confirmed HMPV LRTIs demonstrated acute pneumonia with enlarged type II pneumocytes containing smudged chromatin. Such "smudge cells" are rare

in other paramyxovirus infections (Sumino et al., 2005). The serologic response to HMPV is not fully characterized. In one study of young Japanese children with acute infection, an IgM response was identified in 84% of 26 cases and an IgG response was present in 58% of cases. Of the 14 children who were already IgG positive at the time of diagnosis, 5 had a further rise in HMPV IgG titer (Ebihara et al., 2004). The importance of cell-mediated immunity to virus clearance and recovery from infection is demonstrated by the serious nature of HMPV infections in immunocompromised patients. Study of this response has begun in animal models (Tan et al., 2007).

Laboratory diagnosis of HMPV infection can be accomplished by detection of viral antigen, RNA, or virus culture. Detection of viral RNA by NAT is rapid and sensitive if assays are optimized to detect all four HMPV lineages uniformly (Maertzdorf et al., 2004). Kits are commercially available that can identify HMPV RNA alone or concurrently with other respiratory viruses (Nolte et al., 2007; Aslanzadeh et al., 2008; Pabbaraju et al., 2008). Tube culture is considerably less sensitive and often requires longer than 2 weeks before CPE is evident. Vero or LLC-MK2 monkey kidney cells, as well as other cell types, can be used (Ingram et al., 2006). A low concentration of trypsin in the culture medium may enhance recovery in some cell types. The CPE can be subtle and varies from focal rounding to syncytia formation, as with RSV (van den Hoogen et al., 2001; Tang and Crowe, 2007). Monoclonal antibodies are commercially available for detection of viral antigen by IF in centrifugation-enhanced culture or direct specimens. Centrifugation culture detected 77% of specimens positive by NAT after 2 days of inoculation in one study (Landry et al., 2005). Sensitivity of IF in respiratory tract cells ranges from 62% to 95% compared to NAT (Aslanzadeh et al., 2008; Vinh et al., 2008). Monoclonal antibodies have also been used for virus typing (Gerna et al., 2006b). Other HMPV antigen assays include immunochromatography and EIA, although data on their clinical accuracy are limited at present (Kikuta et al., 2007). Serology is of limited diagnostic usefulness because a convalescent-phase specimen is required for maximum sensitivity. Several serologic assays have been developed in IF or EIA formats and used for epidemiologic studies (Ebihara et al., 2004; Leung et al., 2005b).

At the present time, there are no approved or licensed antiviral or prophylactic treatments for HMPV. Ribavirin has antiviral activities against HMPV in vitro and in animal models, but there are only anecdotal reports of clinical use. Severe HMPV pneumonia in a lung transplant recipient was treated successfully with ribavirin according to one report (Raza et al., 2007). Glucocorticoids demonstrate no such benefit (Wyde et al., 2003; Hamelin et al., 2006). Several sulfonated compounds such as heparin or NMSO3 inhibit early stages of HMPV replication but have not been tried clinically (Wyde et al., 2004). The benefit of passive immunoprophylaxis in prevention of RSV disease in high-risk groups has prompted similar investigations for HMPV. One monoclonal antibody directed against the HMPV F protein attenuates HMPV disease in mice (Hamelin et al., 2008). No vaccine for HMPV is currently available or in clinical trials, but several candidates, including live attenuated, virus-vectored, and subunit vaccines, are being pursued (Herfst and Fouchier, 2008).

Parainfluenza Virus

The parainfluenza viruses are the most significant cause of croup worldwide and are second only to RSV (or perhaps HMPV) as a cause of viral bronchiolitis and pneumonia in infants and young children. In the United States alone, from 6,000 to almost 30,000 children are hospitalized annually due to parainfluenza virus-associated respiratory tract disease (Counihan, 2001). Isolates of these viruses were initially obtained in the 1950s from cultures demonstrating CPE or hemadsorption when inoculated with respiratory tract specimens from children and adults with acute respiratory illness. Their name reflects the early observations that some of the symptoms induced by these viruses are influenza-like and that the particles of both viruses have hemagglutination and NA activities. Subsequently, parainfluenza and influenza viruses were found to display considerable structural and functional dissimilarity and were classified in different virus families.

The parainfluenza viruses belong to the *Paramyxovirinae* subfamily of the family *Paramyxoviridae* (Table 1). Viruses in this subfamily differ from members of the *Pneumovirinae* subfamily, such as RSV and HMPV, in several ways. The most notable difference is that the parainfluenza viruses contain an attachment molecule with combined hemagglutination and NA activities that members of the *Pneumovirinae* subfamily lack (Chanock, 2001). Parainfluenza virions are of medium size, 150 to 200 nm in diameter, enveloped, and roughly spherical in shape, although filamentous forms occur. They contain a nonsegmented, single-stranded, negative-sense RNA genome of approximately 15.5 kb that encodes a basal complement of six structural proteins. An N encases the RNA, forming the helical nucleocapsid. The L protein and P protein form the polymerase complex. The M protein connects the nucleocapsid to two surface glycoproteins, HN and F, which project through the viral envelope and form short spikes. The HN protein confers the hemagglutinating (sialic acid binding) and NA (sialic acid receptor cleaving) activities. Functions of HN include mediating virus attachment to sialic acid-containing cellular receptors, triggering of the F protein to initiate fusion between virus and host cells, and cleaving sialic acid residues from residual receptors on the viral envelope so that particles do not self-aggregate during release from infected cells (Suzuki et al., 2001; Porotto et al., 2005). The HN and F proteins are the major antigenic determinants.

There are four parainfluenza virus types which are further categorized into two genera based primarily on differences in genetic organization and antigenicity. Types 1 and 3 are grouped within the *Respirovirus* genus, whereas types 2 and 4 are in the *Rublavirus* genus. The two genera also differ in their complement of the accessory genes V, D, and SH. These proteins are nonessential for virus replication in vitro but may be important in viral fitness or immune system evasion (Nagai and Kato, 2004). Parainfluenza virus type 4 is further classified into subtypes 4A and 4B based on differences in antigenicity and organization of the P and HN genes (Killgore and Dowdle, 1970; Kondo et al., 1990; Komada et al., 2000). Differentiating between these two subtypes is of uncertain clinical relevance at present. Polymorphisms occur within each parainfluenza virus type but seem to be of little importance to virus evolution. Such variants, however, can affect the performance of diagnostic assays and may complicate vaccine design (Swierkosz et al., 1995; Henrickson and Savatski, 1996).

Parainfluenza virus infections are acquired like other paramyxovirus infections, by inoculation of mucous membranes of the respiratory tract with infectious secretions from fomites or large-droplet aerosols. After an incubation period of 2 to 6 days, replication can be detected in nasopharyngeal

cells and symptoms appear. Shedding continues for 3 to 10 days thereafter but can be quite prolonged in some asymptomatic immune-normal individuals, immunocompromised patients, or persons with chronic respiratory disease (Muchmore et al., 1981; Chanock et al., 2001; Peck et al., 2007). Survival of virus on fingers is brief, although infectivity can last up to 10 h on surfaces. This hardiness, a low infectious dose (approximately 80 50% tissue culture infectious doses for type 1), and prolonged shedding contribute to efficient transmission to susceptible individuals and frequent nosocomial spread (Brady et al., 1990; Smith et al., 1966; Ansari et al., 1991). Indeed, nosocomial parainfluenza virus infections rank as a top cause for closure of medical facilities (Hansen et al., 2007).

Primary infections occur during infancy or early childhood, with most individuals infected by 5 years of age. URTIs are the most common clinical manifestation. In young subjects, parainfluenza viruses rank as the third or fourth most common cause of URTIs when comprehensive molecular assays are used (Legg et al., 2005; Regamey et al., 2008). Most of these illnesses are mild and self-limited, although otitis media can complicate up to a third of cases in infants and young children (Chonmaitree et al., 2008). Common clinical features include rhinitis, bronchitis, pharyngitis, and cough. Fever lasting 2 to 3 days can occur in up to three-fourths of cases. No signs or symptoms clearly distinguish URTIs caused by the four parainfluenza virus serotypes or other respiratory viruses.

Approximately 15% of these illnesses spread to the lower airways (Reed et al., 1997). Persistent fever and productive cough usually signal this involvement. The signature manifestation of parainfluenza virus LRTI is croup, with its characteristic barking cough identifying airway obstruction. Type 1 causes about half of all cases of croup; types 2 and 3 cause a smaller but appreciable proportion. Parainfluenza virus type 1-associated croup typically occurs in children between 6 months and 6 years of age during epidemics in the autumns of odd-numbered years. As many as 250,000 U.S. children are probably affected during such outbreaks (Marx et al., 1997; Henrickson et al., 2004). The illness is usually self-limited. Some children develop progressive airway obstruction and require supportive care, but most cases can be managed without hospitalization.

On the other hand, LRTIs due to type 3 are more endemic and tend to present in a younger age group, mostly during the first year of life or soon thereafter. Bronchiolitis or pneumonia is the most frequent clinical presentation (Henrickson, 1998; Fry et al., 2006). As many as 20,000 U.S. neonates and young infants acquire symptomatic parainfluenza virus type 3 infections annually, many of whom are hospitalized. Thus type 3 is responsible for most cases of severe disease. Wheezing, tachypnea, retractions, and cyanosis are common clinical features. Overall, nearly 14% of all hospitalizations for LRTIs in U.S. children under 5 years of age are attributable to parainfluenza virus types 1, 2, or 3. Most of these infections are self-limited, but poor outcomes can occur in young patients or individuals with underlying medical problems. Most fatalities are associated with type 3 (Henrickson et al., 2004). Spread beyond the respiratory tract is rare, but parainfluenza virus-associated parotitis, aseptic meningitis, and encephalitis are reported (Zollar and Mufson, 1970; Arguedas et al., 1990; McCarthy et al., 1990). Fatal infections involving gastrointestinal and urinary tract tissues have occurred in children with congenital immunodeficiencies (Madden et al., 2004).

Somewhat less is known about parainfluenza type 4 because the virus is difficult to culture. Seroprevalance studies indicate that such infections are not rare. In one study, significant levels of maternally derived type 4 antibodies were found in 60 to 84% of infants, whose titers then declined. About 50% of these children acquired antibody by 5 years of age, and by adulthood, 75 to 95% of subjects were seropositive (Gardner, 1969). All syndromes induced by other parainfluenza viruses are probably caused by type 4 (Lindquist et al., 1997; Slavin et al., 2000). Recent studies using NAT suggest that type 4 infections may be underdiagnosed and that some infections are clinically significant. For example, one study of LRTIs in pediatric patients identified almost 10-fold-more type 4 infections by NAT than by culture, resulting in more illnesses attributable to type 4 than to type 2 (Aguilar et al., 2000). In one large outbreak of type 4 respiratory disease identified by molecular testing, 38 institutionalized children and 3 staff members were affected. Serious LRTIs occurred in 7% of patients, one of whom required ventilatory support and intensive care (Lau et al., 2005).

Reinfections with parainfluenza viruses are frequent, especially during the first two years of life, most likely due to an incomplete and transient immune response (Glezen et al., 1984). The primary infection induces an IgM response. Ample titers to the HN protein occur, but responses to the F protein are generally weak. Reinfection induces a more durable antibody response with higher titers to both proteins. This subsequent response and the amount of secretory IgA in the mucous membranes correlate best with protective immunity. The importance of the cellular immune response is demonstrated by serious and persistent parainfluenza virus infections in immunocompromised individuals, but information on this aspect of the immune response to parainfluenza virus remains limited. In otherwise healthy adults, reinfections with parainfluenza virus account for 1 to 15% of all acute respiratory illnesses. Most reinfections manifest as mild URTIs, although lower airway involvement can occur. In one recent study of hospitalized school-age children with pneumonia, for example, viruses were identified by NAT in 65% of cases, with parainfluenza viruses responsible for 12% of virus-positive cases (Tsolia et al., 2004). Parainfluenza viruses were among the four most common viral pathogens in hospitalized adults in other studies (Marx et al., 1999; Angeles et al., 2006). They are less frequently associated with exacerbations of asthma or COPD than rhinoviruses or CoVs (Beckham et al., 2005; Khetsuriani et al., 2007; Ko et al., 2007).

Parainfluenza virus infections in the elderly have been less well studied than RSV infections. Swedish investigators found serologic evidence of infection in 11% of community-dwelling elderly with pneumonia and 7% of those hospitalized with acute respiratory illnesses (Fransen et al., 1969). Prospective studies in nursing homes documented parainfluenza virus infections in 2 to 14% of residents, with these viruses of lesser significance than rhinovirus, CoV, and influenza virus infections (Graat et al., 2003). One notable type 3 outbreak in an elder care facility, however, involved four fatalities (Todd et al., 2000). Parainfluenza viruses may also predispose patients to bacterial superinfection, perhaps related to viral NA activity, which improves adherence of bacteria to infected mucosa (Avadhanula et al., 2006). Indeed, parainfluenza virus was implicated as a precursor to a nursing home outbreak of invasive pneumococcal disease (Falsey and Walsh, 2006). Initial symptoms of infection in the elderly are typically nondistinctive and may include fever, rhinorrhea, cough, and sore throat. An influenza-like syndrome has also been described (Hui et al., 2008).

Parainfluenza virus infections are reported in 2 to 7% of adult and pediatric HSCT patients in most series, with type 3 most often involved (Kim et al., 2007). Upper airway symptoms usually predominate. In one large series, 10% of URTIs progressed to involve the lower airways (Nichols et al., 2001a). These LRTIs, however, can persist for months and can be associated with serious airflow decline. Mortality rates from 4 to 75% have been reported (Erard et al., 2006). Unlike RSV infections, many parainfluenza virus-infected HSCT patients are coinfected with other pathogens, which may contribute to the wide range of outcomes observed (Lewis et al., 1996). Asymptomatic shedding is frequent and may induce or perpetuate nosocomial infections (Cortez et al., 2001; Peck et al., 2007). One particularly stubborn outbreak of parainfluenza virus type 3 among HSCT outpatients lasted over 10 months and was attributed to persistence of virus on environmental surfaces and asymptomatic shedding by both patients and staff (Nichols et al., 2004a). Lung transplant recipients may acquire significant parainfluenza virus infections as well. The incidence of such infections at one large transplant center was 5.3 per 100 patients, with lower airway involvement in 10 to 66% of cases. Acute graft rejection or bronchiolitis obliterans may result (Vilchez et al., 2003; Khalifah et al., 2004; Kumar et al., 2005). Parainfluenza virus infections with lower airway involvement are also reported in subjects with HIV infection or cancer (Mendoza Sanchez et al., 2006; Garbino et al., 2008).

Infections are typically diagnosed by viral isolation in culture, antigen detection, and most recently, NAT. Parainfluenza virus can be isolated in primary or continuous monkey kidney cells. Growth is slow, with 3 to 5 days or longer required. A low concentration of trypsin in the culture medium may improve recovery. Replication is detected by CPE, which differs subtly by serotype, or by hemadsorption of guinea pig red blood cells to infected monolayers. Type 4 can require 10 days or more to express CPE, and hemadsorption may be weak. Confirmation is recommended and can be performed by IF of infected cells with commercially available group- or type-specific antibodies. Centrifugation-enhanced cultures using human A549 or mixed cells can detect 60 to 90% or more of type 1, 2, or 3 positive specimens in 1 to 2 days (Rabalais et al., 1992; LaSala et al., 2007). Rapid detection of virus antigen by IF in exfoliated cells is more sensitive than culture, but testing by NAT can increase the detection rate substantially (Landry and Ferguson, 2000). For example, a 41% increase in parainfluenza virus yield using NAT compared to culture was reported with nasal or throat swabs from ill children (Weinberg et al., 2004). Likewise NAT detected 30% more type 3, 64% more type 1, and 88% more type 2 than IF in specimens from pediatric patients (Kuypers et al., 2006). A multiplex molecular assay that includes and discriminates among the 3 major virus types is now available and approved for diagnostic purposes in the United States (Pabbaraju et al., 2008). Serology is of little importance for clinical use due to the requirement for acute- and convalescent-phase specimens. Serologic cross-reactions between the parainfluenza viruses and mumps virus can also hamper interpretation of results.

No proven antiviral treatment for parainfluenza virus infections is available. Ribavirin has activity against the virus in vitro and in animal models but is of uncertain benefit in humans. Reports of its use are mostly limited to HSCT or lung transplant recipients and are anecdotal in nature as well as variable in outcome. For example, little improvement in mortality was demonstrated in over 40 parainfluenza virus type 3-infected HSCT recipients treated with ribavirin at two large U.S. transplant centers compared to controls (Kim et al., 2007). Similarly, only minimal benefit was demonstrated in 5 parainfluenza virus type 3-infected lung transplant recipients (McCurdy et al., 2003). In contrast, ribavirin treatment of parainfluenza virus type 3 infections in a cardiac transplant patient, a child with severe combined immunodeficiency disease, and early treatment in HSCT patients appeared to be beneficial (Wright and O'Driscoll, 2005; Frank et al., 1983; Dignan et al., 2006). Therefore, the efficacy of ribavirin remains uncertain. Other promising treatments include NA inhibitors, activators of interferon, polyoxometalates, and siRNAs (Alymova et al., 2004; Bitko et al., 2005; Shigeta et al., 2006; Thakur et al., 2007). Dexamethasone and corticosteroids are established treatment for symptomatic relief of severe croup (Leung et al., 2004). However, corticosteroid use in parainfluenza virus-infected HSCT patients is associated with the development of viral pneumonia (Nichols et al., 2001a).

Currently, no parainfluenza virus vaccines are licensed. Most efforts have been directed toward developing a vaccine for type 3, although vaccines against type 1 and formulations effective against all 3 types are under consideration. Development has been slowed by tactical questions of whether a stand-alone vaccine is indicated, whether the effort should be directed toward a combined parainfluenza virus type 3-RSV vaccine, or if vaccination against parainfluenza viruses should await proof of principle that an RSV vaccine will work. Current efforts are focused on intranasally administered vaccines using the classical technique of cold-passage, host range attenuation, or reverse genetics to introduce specific attenuating mutations (Schmidt, 2007). The vaccine that is farthest advanced is a cp-45, a cold-passaged virus (Belshe et al., 2004b). Trials are under way or planned with a chimeric vaccine of type 3 HN and F genes inserted into a bovine parainfluenza virus type 3, a combined RSV-parainfluenza virus vaccine, and a parainfluenza virus type 1 vaccine (Schmidt et al., 2000; Belshe et al., 2004a; Bartlett et al., 2007).

Rhinovirus

Rhinoviruses comprise the *Rhinovirus* genus of the *Picornaviridae* family (Table 1). The name originates from their predominant replication in the nose. Rhinoviruses cause more common colds (minor URTIs) than any other virus infecting the upper respiratory tract. The first isolate was obtained in the mid-1950s from nasopharyngeal washings of individuals with colds. Additional recoveries were made by mimicking conditions in the "nose," including reducing the culture temperature to 33°C, lowering the pH to neutral, and aeration of culture tubes by gentle rolling.

More than 100 distinct rhinovirus serotypes are currently defined by neutralization tests. They have been classified several ways. One system identifies 2 species, human rhinovirus A (n = 75 serotypes) and human rhinovirus B (n = 25 serotypes) based on susceptibility to capsid-binding antivirals. This grouping is confirmed by phylogenetic analysis of capsid protein sequences (Ledford et al., 2004). A third species, human rhinovirus C, has been proposed to encompass novel strains recently identified only by molecular analysis. Members of this species appear to cause more serious illness than typically associated with rhinoviruses. They were discovered during an investigation of an outbreak of influenza-like illnesses in New York and are therefore commonly called "Rhinovirus New York" (Lamson et al., 2006). Similar viruses have now been identified in children with LRTIs in Australia, Europe, and the United States (McErlean et al., 2007; Dominguez et al., 2008). A second classification system

based on cellular receptor usage categorizes the viruses differently. There is a major group (n = 88 serotypes) which uses the ICAM-1 (intracellular adhesion molecule 1) receptor and a minor group (n = 12 serotypes) which uses low-density lipoprotein. Rhinovirus 87 does not fit into either classification system and is now considered to be the acid-sensitive enterovirus type 68.

The rhinovirus virion is a small (15 to 30 nm), nonenveloped, icosahedral particle encasing a single-stranded, positive-sense RNA of approximately 7.2 kb. Its RNA contains a noncoding region at the 5′ end and a single open reading frame. The capsid contains four proteins, VP1, VP2, VP3, and VP4, which are created by posttranslational cleavage of a precursor polyprotein. There are also several small nonstructural proteins, including 2 proteases and an RNA-dependent RNA polymerase. The VP1, VP2, and VP3 proteins are on the surface of the capsid; VP4 is on the inside and helps anchor the RNA core to the capsid. The capsid surface contains "canyons" that are important for binding to cellular receptors. Variation in the surface proteins leads to antigenic diversity and stimulates type-specific host immune responses. There is no group antigen. Unlike enteroviruses, most rhinovirus serotypes lose infectivity upon exposure to mild acid, accounting for their failure to infect the gut.

Rhinoviruses are the most frequent cause of the common cold in all age groups. Typically, about half of all colds are attributable to the virus, but up to 80% of URTIs may be rhinovirus associated during periods of increased incidence. Infants, young children, and the elderly are especially susceptible (Arruda et al., 1997; Graat et al., 2003; Legg et al., 2005). Even though most colds are trivial, their sheer number produces more episodes of illness, greater restriction of activities, and more physician visits than any other human pathogen (Monto et al., 2001). Upper airway complications of rhinovirus-associated colds are increasingly recognized when molecular assays are used. Acute otitis media can develop in up to a third of rhinovirus URTIs or asymptomatic infections in young children (Winther et al., 2006; Chonmaitree et al., 2008). Acute and chronic sinusitis are frequent sequellae of rhinovirus URTIs as well (Pitkaranta et al., 2001; Jang et al., 2006).

Although rhinoviruses are traditionally associated with URTIs, they can also cause lower airway disease with considerable morbidity. This association is most obvious when molecular methods and lower airway tissue or fluids are used (Garbino et al., 2004a; Mosser et al., 2005). Rhinovirus LRTIs are most frequent in young children, in persons with underlying medical conditions, and the elderly (Hayden, 2004; van Gageldonk-Lafeber et al., 2005). In one recent surveillance study, rhinoviruses caused nearly 5 hospitalizations per 1,000 children less than 5 years of age, with pneumonia occurring in 16% of rhinovirus-positive patients. The highest hospitalization rates were in children with a history of wheezing or asthma (Miller et al., 2007). In the elderly, the burden of rhinovirus LRTI may exceed that of influenza virus. Other rhinovirus-associated LRTIs include croup, tracheobronchitis, bronchiolitis, and the aforementioned influenza-like syndrome (Hayden, 2004; Choi et al., 2006; Renwick et al., 2007). In some studies, rhinoviruses were second only to RSV as a cause of acute bronchiolitis. Rhinoviral bronchiolitis in infancy may predispose to asthma in later life (Heymann et al., 2005; Lemanske et al., 2005). Fatalities due to rhinoviruses in immune-normal individuals are infrequent.

Rhinoviruses may be the major virus associated with exacerbations of pre-existing pulmonary conditions such as asthma, COPD, and cystic fibrosis. Analysis of these studies, however, is complicated by the high rate of rhinovirus detection in asymptomatic subjects by NAT (Beckham et al., 2005; Khetsuriani et al., 2007; Wat et al., 2008). Highly immunocompromised patients, particularly HSCT recipients, often acquire serious rhinovirus LRTIs, especially if infected prior to engraftment. Frequent coinfections, prolonged shedding, and high morbidity result, but the significance of rhinovirus in causing direct pulmonary damage, in predisposing to secondary bacterial invaders, or causing death remains unclear in this complex population (Ison et al., 2003; van Kraaij et al., 2005). Rhinovirus infections in lung transplant patients can predispose patients to graft rejection (Kumar et al., 2005; Kaiser et al., 2006). Infections in other SOT recipients are usually mild (Kim et al., 2007). Fatalities in some immunocompromised patients are documented (Gutman et al., 2007). An especially high frequency (8% to over 50%) of coinfections with rhinovirus and other respiratory viruses occurs in patients with acute respiratory tract infections (Legg et al., 2005; Weigl et al., 2007). The importance of rhinoviruses in enhancing the severity of LRTIs caused by other pathogens and in exacerbations of COPD remains to be determined.

Rhinovirus URTIs usually begin by autoinoculation of virus into the nose or eyes. Aerosol transmission may occur with prolonged contact. Psychological stress may increase the occurrence of symptomatic infections (Douglas, 1967; Takkouche et al., 2001). The incubation period is approximately 2 to 3 days after exposure, but symptoms can appear up to 7 days. Illness usually lasts 10 days to 2 weeks. It begins with a profuse watery nasal discharge which may become mucopurulent and viscous. Other symptoms may include malaise, nasal congestion, sneezing, mild headache, pharyngitis, and cough. Fever is infrequent. Peak viral shedding occurs from the nose at 2 to 3 days. Shedding usually ceases by 7 to 10 days, but infectious virus can be recovered for up to 3 weeks in some individuals. Viral RNA can persist even longer (Landry, 2007; Regamey et al., 2008). Replication occurs in the cytoplasm of nasal mucosal cells, but there is surprisingly little tissue damage. Symptoms largely derive from induction of inflammatory mediators (Message and Johnston, 2001). Type-specific immunity consists of IgG neutralizing antibodies in serum and a local IgA response which is probably most important for long-lasting immunity. Interferon and other innate responses likely contribute to recovery as well because individuals who have one rhinovirus infection rarely get another within the next month. Asymptomatic infections are frequent and may occur in 20% or more of healthy individuals (van Gageldonk-Lafeber et al., 2005; Wright et al., 2007).

Diagnosis of rhinovirus URTIs is usually unnecessary for clinical purposes, but a nasal specimen is preferred when diagnosis is required because virus titer is usually highest in the nose. Rhinoviruses can also be recovered from NP specimens, bronchoalveolar lavage fluid, or lung tissue; sputum and tracheal aspirates usually have a low yield. Culture is typically performed on human cells such as MRC-5 or fetal tonsil. Conditions should simulate those in the nose, i.e., pH of 7.0, temperature of 33 to 35°C, and gentle rolling of tubes. Typical CPE in fibroblasts include large and small round, refractile cells appearing 1 to 4 days after inoculation or longer, depending on serotype and inoculum. The effect may regress or slowly evolve. Some isolates can be presumptively identified by their appearance but others may resemble enterovirus. Sensitivity to a brief acid treatment can distinguish rhinoviruses from the acid-stable enteroviruses,

but the assay is cumbersome and infrequently performed by clinical laboratories (Landry, 2007). Identification can also be performed by virus neutralization using type-specific antibody, sequence analysis of conserved regions in viral genes, or other molecular assays. No rhinovirus-specific monoclonal antibodies are available for viral antigen detection by IF, nor are commercially available antigen detection assays available due to the lack of a group antigen. The large number of serotypes and lack of a common antigen also limits use of serology to research laboratories.

Molecular methods are increasingly used for rhinovirus detection and diagnosis because they are significantly more rapid and sensitive than culture. Many of these assays, including a commercially available NAT that detects rhinovirus and other respiratory viruses, identify a region in the 5′ untranslated region shared by rhinoviruses and enteroviruses without differentiating between them (Pabbaraju et al., 2008). Assays designed to detect only rhinoviruses may not detect all serotypes or require some form of postamplification processing to detect all serotypes (Miller et al., 2007; Wright et al., 2007). At least one recently described NAT for rhinovirus may not require such manipulations (Lu et al., 2008).

Antiviral therapy for rhinovirus infections would have widespread application, but none is currently approved or available. Many promising compounds have been evaluated, including substances blocking virus attachment, uncoating, RNA replication, and protein synthesis. Compounds interfering with cellular susceptibility and immunomodulators have also been assessed. Many putative antivirals show activity in the laboratory, but issues of drug delivery often reduce their clinical benefit. Among the compounds tried are soluble ICAM-1, inhibitors of ICAM-1 up-regulation, interferon alpha-2, intranasal imiquimod, intranasal ipratropium bromide, and pyrrolidine dithiocarbamate (Turner, 2005). One especially promising substance was pleconaril, which binds to a pocket in the VP1 protein and blocks uncoating of most picornavirus serotypes (Ledford et al., 2005). It was not approved for use in humans due to interference of the compound with the efficacy of oral contraceptives. In volunteer trials, a viral protease inhibitor, ruprintrivir, was effective but caused nasal irritation. Alternative substances such as echinacea have been evaluated, but most trials are inconclusive due to poor controls or virology that was not thorough (Hulisz, 2004; Schoop et al., 2006). Oral zinc sulfate may have some benefit for prevention and treatment of rhinovirus infections in children (Kurugol et al., 2006). Currently, prevention of infection and symptomatic relief are the most effective interventions.

CoVs

CoVs were once considered rather harmless, human RNA viruses associated with colds and an occasional LRTI. Animal CoVs, on the other hand, cause a wide variety of different diseases in mammals and birds. A novel virus now known as the SARS-CoV then crossed from animals into humans in 2002, creating a global outbreak of respiratory disease with considerable morbidity and mortality. This event greatly intensified interest in the CoVs and the complex relationships between animals, humans, and viruses that permits emergence of new human respiratory pathogens.

Currently, five CoV types are known to cause respiratory disease in humans. The first isolates were identified in the 1960s from nasal secretions of adults with mild URTIs but grew only in human embryonic tracheal organ cultures and were lost to follow-up. Serologic studies demonstrated that these viruses were antigenically related but distinct from two subsequently discovered CoVs, 229E and OC43, which were successfully adapted to cell culture and therefore best studied. The SARS-CoV was the third CoV identified. The fourth CoV, NL63, was reported from children with acute respiratory symptoms in The Netherlands in 2004; a nearly identical NH strain was identified in U.S. children shortly thereafter (Fouchier et al., 2004; van der Hoek et al., 2004; Esper et al., 2005b). The fifth human CoV, HKU1, was recovered from elderly patients with LRTIs in Hong Kong (Woo et al., 2005).

The *Coronaviridae* family is composed of medium-sized, enveloped, positive-strand RNA viruses. There are two genera. Members of the *Torovirus* genus cause gastroenteritis in humans. The *Coronavirus* genus contains animal as well as human respiratory CoVs which are classified into 3 groups based on genetic and antigenic differences (Table 1). Viruses of group I and group II affect humans and other mammals. Group I viruses of human significance are 229E and NL63. Group II contains OC43, HKU1, and SARS-CoV, although some experts believe that the SARS-CoV should form a separate group (Snijder et al., 2003; Vijaykrishna et al., 2007). Group III viruses infect only birds. Receptor use differs among the different human CoVs and is probably involved in pathogenesis. Angiotensin-converting enzyme 2 (ACE2) is used by NL-63 and SARS-CoV. This protein is present on the surface of ciliated airway epithelial cells, endothelial cells, and gastrointestinal epithelium. Human aminopeptidase N (CD13), the receptor for 229E, is found on human myeloid, intestinal, lung, and kidney cells. The HLA class I antigen is a receptor for OC43. The receptor for HKU1 is not yet known (Pyrc et al., 2007).

The CoVs are pleomorphic, roughly spherical particles 80 to 160 nm in diameter. Each virion contains a helical nucleocapsid surrounding a genome of approximately 27 to 30 kb, the largest among RNA viruses. The viral RNA-dependent RNA polymerase is quite error prone and frequently switches strands during replication, inducing high rates of mutation and frequent recombination during mixed infection. This property results in clinical isolates with considerable genetic variation (Moes et al., 2005; Gerna et al., 2006b). The viral genome encodes a single polyprotein that is cleaved by viral proteases into the various proteins. The 5′ two-thirds of the virus genome specify the nonstructural proteins necessary for RNA replication and several accessory proteins. The 3′ third of the genome encodes the structural spike, envelope, and membrane proteins. An HA esterase found only in the group II CoVs was likely introduced into an ancestral CoV from influenza C (Zeng et al., 2008). Projecting from viral envelope are club-shaped, spike proteins that create a "corona" or crown-like appearance of virus particles. Following attachment of these spike proteins to specific cell surface receptors, the virus enters the cell and replicates in the cytoplasm.

The four common CoVs, 229E, OC43, NL63, and HKU1, appear to have circulated in humans for centuries (Pyrc et al., 2006). They also cause milder disease than does the SARS-CoV, which is described in further detail below. The most frequent clinical manifestation of 229E and OC43 is the common cold. Overall 5 to 15% of colds in all age groups worldwide are caused by 229E and OC43; therefore, they are the second most frequent cause of colds, behind rhinoviruses. Most individuals have been infected with one type or the other before 6 years of age (Monto and Lim, 1974; Graat et al., 2003; van Gageldonk-Lafeber et al., 2005; Regamey et al., 2008). Colds induced by 229E or OC42 in otherwise-healthy individuals are usually benign and self-limited, but

somewhat more severe than those caused by rhinoviruses. Typical clinical features include coryza, rhinorrhea, nasal congestion, sore throat, and pharyngeal edema. Wheeze and cough were frequent in a recent prospective study of infants (Regamey et al., 2008). Fever may be absent. Otitis media can complicate up to half of CoV-associated UTIs in young children (Chonmaitree et al., 2008). Infections with 229E or OC43 can also exacerbate asthma, cystic fibrosis, or COPD, although rhinoviruses may be more significant in this regard (Beckham et al., 2005; Khetsuriani et al., 2007; Wat et al., 2008).

Most 229E and OC43 acute respiratory illnesses are acquired by contact or inhalation of respiratory droplets from another infected individual. Transmission is facilitated by virus stability up to 3 h on solid surfaces and 6 days in aqueous solutions (Sizun et al., 2000). The incubation period is 2 to 5 days. Like most respiratory viruses, infectivity is greatest early in the illness. Upper airway symptoms typically last a week but can range from 3 to 18 days. Virus shedding often persists 3 weeks or more after onset and can be detected after symptoms abate (Regamey et al., 2008). Type-specific antibody appears and lasts for a mean of 4 months; there is no heterotypic antibody response. These factors likely contribute to the high reinfection rate (Macnaughton, 1982). The importance of cell-mediated immunity in recovery is unknown. Asymptomatic infections are frequent (Monto and Lim, 1974; van Gageldonk-Lafeber et al., 2005).

The newer NL63 and HKU1 CoVs may be as common as 229E or OC43 and have been identified in all countries where sought. They appear to be responsible for 1 to 10% of all acute airway illnesses. Infections are detected in all age groups. Most initial NL63 infections occur in children under 6 years of age; comparable data for HKU1 are still limited. Upper and lower airways can be involved. Symptoms of NL63- and HKU1-associated URTIs include fever, cough, sore throat, or rhinitis. Lower airway syndromes include croup, bronchitis, bronchiolitis, and pneumonia (Bastien et al., 2005; Esper et al., 2006). The link between NL63 and croup is especially strong. A large prospective study of German children less than 3 years of age found croup in 45% of NL63-infected subjects, compared to only 6% in the control group (van der Hoek et al., 2006). Lower airway involvement is observed most often in children, the elderly, and individuals with underlying chronic diseases. Fatalities are reported and occur mostly at both ends of the age spectrum or in high-risk groups (Bastien et al., 2005; Esper et al., 2006; Garbino et al., 2006). NL63 was responsible for more than 200 hospitalizations per 100,000 children under the age of 6 in one prospective study of children under 18 years of age in Hong Kong (Chiu et al., 2005). Coinfections involving NL63 are especially frequent and are identified mostly in hospitalized patients. The NL63 viral load in single infections is usually higher than in dual infections, suggesting that innate responses to other viruses may suppress CoV replication (Chiu et al., 2005; van der Hoek et al., 2005).

Involvement of 229E and OC43 in LRTI was previously considered uncommon. The availability of NAT that detect and discriminate among CoVs now demonstrates that these two viruses can cause a significant proportion of LRTIs, especially in high-risk patients (Falsey et al., 2002; Garbino et al., 2006; Gerna et al., 2006a). Interestingly, OC43 has predominated in several recent studies of lower airway disease. For example, one prospective study in Switzerland found CoVs in 5.4% of 540 bronchoalveolar lavage specimens from 279 immunosuppressed patients; 11% also contained another respiratory virus. Of the CoV-positive specimens, 41.4% had OC43, 24.1% had 229E, 20.7% had NL63, and 13.8% had HKU1 (Garbino et al., 2006). Another study of hospitalized infants and immunosuppressed patients in Italy, most of whom had LRTIs, identified CoVs in 5.5% of nasopharyngeal aspirates. Again, OC43 predominated and was detected in 53.2% of CoV-positive specimens compared to 229E in 21.3% and NL63 in 19.1%. No HKU1 was identified (Gerna et al., 2006a). Similar results were noted in a Hong Kong study (Lau et al., 2006). Bronchiolitis, an influenza-like illness, and pneumonia are the LRTIs most often reported with these viruses (van Gageldonk-Lafeber et al., 2005; Gerna et al., 2006a).

The common CoVs may also cause disease beyond the respiratory tract. Particles resembling CoV are present in stools of infants and children with diarrhea and other gastrointestinal syndromes but could represent the morphologically similar toroviruses (Payne et al., 1986; Jamieson et al., 1998). Interestingly, six cases of confirmed HKU1 respiratory tract disease were recently described. Three of these patients also had enteric disease, and HKU1 was detected in the stools of two by PCR (Vabret et al., 2006). Hepatitis and seizures out of proportion to body temperature have been reported in a few patients with HKU1 acute respiratory illnesses (Esper et al., 2006; Lau et al., 2006). A role for CoV in multiple sclerosis has been proposed, but the association remains unclear. No evidence of 229E or OC43 infection was found by PCR in one study of brain tissue from 25 persons with multiple sclerosis and 36 individuals without neurologic disease (Dessau et al., 2001). Several reports have linked NL63 with Kawasaki disease, a common childhood vasculitis of unknown cause that is frequently preceded by a viral prodrome (Esper et al., 2005a). Others were unable to confirm this association (Dominguez et al., 2006).

Laboratory detection of the common CoVs was once infrequently performed due to perceived lack of clinical need and paucity of diagnostic approaches. Culture, electron microscopy, and serology are cumbersome and insensitive. Although monoclonal antibodies specific for OC43 and 229E are available, they have been mostly used in research settings (Gerna et al., 2006a; Mahony, 2007). Currently, NAT offers the most practical means for CoV diagnosis. Type-specific primers are often recommended over generic reagents due to genetic variability among CoV types (Gerna et al., 2006a). Kits to detect respiratory viruses by NAT are commercially available for investigational use in the United States (Mahony et al., 2007; Nolte, 2007). Currently there are no specific antiviral treatments or vaccines available for infections due to the common CoVs. This situation may change as further information regarding the frequency and consequences of these infections becomes available.

SARS-CoV

The disease known as SARS was first described in a southern region of China in November 2002. International attention was aroused in early 2003 following the rapid global spread of SARS from an outbreak started by a single patient at a Chinese hotel and a subsequent outbreak in a Hong Kong hospital. Shortly thereafter, a cooperative international effort identified the etiologic agent as a CoV not previously found in humans (Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003). Its association with SARS was proven by fulfilling Koch's postulates in nonhuman primates (Fouchier et al., 2003). Lacking a SARS diagnostic test for most of the pandemic, a case definition was established and used to mount an intense global infection control effort that halted the chain of transmission in July 2003. During the 5-month pandemic,

8,096 human cases and 774 fatalities were reported from 26 countries to the World Health Organization (http://www.who.int/csr/sars/country/table2004_04_21/en/index.html), for a case fatality rate of 9.6%. China and Hong Kong had the most recorded cases, but Canada and the United States had 251 and 27, respectively. The global economic consequence of SARS was estimated at $30 billion to $140 billion.

The progenitor of the SARS-CoV most likely originated from horseshoe bats in China. Bats are a natural reservoir for CoVs in the wild and can harbor a great genetic diversity of these viruses without expressing clinical disease (Wang et al., 2006). Indeed, SARS-like CoVs (SL-CoVs) of several horseshoe bat species in China share sequence homologies of 88 to 92% with the human SARS-CoV. A large region of sequence diversity exists between the SL-CoVs of bat origin and the human SARS-CoV in the portion of the spike gene responsible for attachment to cellular receptors. Mutations in this spike gene region probably allowed the SL-CoV to infect other animal species (Ren et al., 2008). Similar introductions of bat CoVs into different animal species may have preceded the development of other human CoVs as well (Vijaykrishna et al., 2007). It is unlikely that humans were directly infected by bat SL-CoV, however. Intermediate exotic mammals in the vicinity of live-animal markets of China were probably infected initially; subsequent mutations then permitted transmission to humans. Palm civets are the most likely intermediate hosts; over 80% of these animals had evidence of SL-CoV infection in some markets (Tu et al., 2004). The close contact between humans and live animals in wet markets or restaurant settings probably facilitated the initial human infections, followed by human-human transmission. The exact mode of interspecies transmission remains uncertain but most likely involved contact with contaminated feces, urine, blood, or aerosols. Similarly, most human-to-human transmissions occurred via respiratory droplets and direct contact, although fecal aerosols were probably important in the Hong Kong hotel outbreak.

SARS begins with a nonspecific prodrome 2 to 10 days after exposure that includes fever, myalgia, malaise, headache, and often diarrhea. Low white blood cell count with a pronounced lymphopenia and enzyme abnormalities are usually evident. A respiratory phase with dry cough and dyspnea begins 2 to 7 days after illness onset. Radiographic evidence of pneumonia appears by a week to 10 days of illness. Progression to severe acute respiratory distress occurs in 20 to 30% of patients; the remainder begins to recover at the end of the second week or soon thereafter. Case fatality rates of 50% or greater were reported in the elderly or in persons with underlying conditions (Muller et al., 2006). Fatal cases had evidence of virus replication in multiple organs, including lung, bowel, liver, and kidney (Farcas et al., 2005). Disease was uncommon and mild in individuals under 24 years of age (Stockman et al., 2007). Unlike other viral acute respiratory illnesses, the period of maximum infectivity and highest viral loads in upper airways occurs during the second week of illness (Poutanen et al., 2003). This is when most patients are hospitalized and need intensive care. Therefore, transmissibility is low early in the illness, and most cases at the peak of the pandemic were acquired nosocomially. Infections can be asymptomatic or atypical yet transmit disease. Seroconversion is usually demonstrable in weeks 2 to 3 of illness, with IgG and IgM responses appearing together. Occasionally antibodies are detectable in the first week of illness but can appear 28 days or longer after onset.

There is real concern that SARS could reappear. Potential sources for new outbreaks include reintroduction to humans from an animal reservoir, persistent infection in previously ill persons, or the laboratory. Serologic studies indicate that humans in the region were infected with SL-CoVs on multiple occasions prior to the recognition of SARS in 2002. Sporadic cases of SARS-like disease also continued for a time in the postpandemic period, once the ban on exotic animal sales was lifted. Thus independent species-crossing events may occur again depending on the distribution of the reservoirs or transmitting hosts (Liang et al., 2004; Zheng et al., 2004). Three laboratory accidents involving the SARS-CoV also resulted in clinical disease and human-to-human transmission. Surveillance of animal reservoirs and humans therefore continues.

In the event that SARS reappears, accurate detection of initial cases would be especially challenging. To avoid overtesting, generating false-positive results, and creating unnecessary alarm, a case definition has been developed that focuses on patients with epidemiologic risk factors, clinical presentation, and lack of an alternative explanation. Unusual clusters of unexplained pneumonia, particularly in a laboratory or hospital, may also be suspicious. Laboratory confirmation of clinically suspect cases is essential, given the nonspecific nature of initial symptoms. Such testing is now available in U.S. state public health and some reference laboratories and is recommended if no alternative diagnosis is established after 72 h in those considered at high risk. Due to the low positive predictive value of test results in the current absence of circulating SARS-CoV, public health officials should be consulted before testing.

Detection of SARS-CoV RNA by NAT is currently the most useful diagnostic test. Although molecular assays had low sensitivity during the pandemic before day 5 or after day 15 of illness, values approaching 80% can now be achieved on day 3 with newer amplification systems and better extraction methods (Mahony and Richardson, 2005). During the first week of illness, NP and throat swabs plus a serum or plasma specimen should be sent for laboratory testing. After the first week, these specimens plus stool should be submitted again. The slow development of the antibody response limits the immediate usefulness of serology. Therefore, serum should be collected when the diagnosis is initially suspected and at later times, if indicated. A variety of serologic approaches such as IF or EIA can be used; positive results should be confirmed by neutralization. Although NAT is the most sensitive diagnostic approach, the virus can also be grown on the Vero E6 monkey cell line, which displays a rapidly progressive CPE of cell rounding, refractivity, and detachment (Ksiazek et al., 2003). Culture should not be attempted by routine clinical virology laboratories because BSL-3 conditions are required for safe handling. Further assistance with clinical and laboratory evaluation and the current case definition are available in a guidance document on the CDC's SARS website at http://www.cdc.gov/ncidod/sars/absenceofsars.htm.

Many attempts were made to reduce virus replication or modulate the immune response during the pandemic, but few large studies were conducted and none was controlled. Compounds used most often were ribavirin, due to its broad-spectrum activity against both DNA and RNA viruses, and corticosteroids. Ribavirin initially appeared promising, but larger trials suggested lack of efficacy, with frequent and serious side effects (Booth et al., 2003). It was also ineffective at clinically achievable doses against SARS-CoV in vitro and actually enhances virus replication in mice (Barnard et al., 2006). Corticosteroids were mostly used in combination with ribavirin, but results were conflicting and side effects were

problematic (Wang et al., 2003a). The protease inhibitor cocktail lopinavir-ritonavir has activity against SARS-CoV in vitro. It significantly reduced mortality when used with ribavirin and corticosteroids as initial treatment or rescue therapy in some studies (Chu et al., 2004). Other modalities assessed in fewer patients with no conclusive efficacy data included interferon alpha, intravenous gamma globulin, convalescent-phase serum, exchange transfusion, and traditional Chinese medications. Continued research has identified several candidate compounds targeting different stages of the virus life cycle and the immune response (Haagmans and Osterhaus, 2006). To date, there is still no vaccine and no effective commercially available drug for treatment of SARS.

Adenovirus

The adenoviruses are medium-sized (70 to 90 nm), nonenveloped, DNA viruses belonging to the family *Adenoviridae*. They are widely distributed in nature, but only members of the *Mastadenovirus* genus infect humans. The human adenoviruses cause a broad spectrum of disease with respiratory tract and gastrointestinal tract infections being the most common. Considerable interest in these viruses revolves around their ability to introduce genes into human cells (Jager and Ehrhardt, 2007). The name acknowledges their original isolation from cultures of human tonsils and adenoids in the early 1950s. Currently 51 human adenovirus types have been identified (de Jong et al., 1999). They are grouped into six species designated A to F, with species B further subdivided into two subspecies, based on several classification schemes (Table 4).

The adenovirus virion contains a single, linear, double-stranded DNA molecule of about 36 kb that encodes approximately 40 genes. The genome is relatively stable, and recombination events are rare. The icosahedral capsid is formed by two types of capsomeres called hexons or pentons. Hexons form the particle's 20 triangular faces, with one penton at each of 12 vertices. From each penton projects a fiber (two in some enteric serotypes) and a terminal knob that aids attachment to cellular receptors. The hexon contains antigenic sites common to all adenoviruses, but these sites are sequestered within the capsid, so genus-specific antibodies do not form. Species-specific determinants are located on the fiber, which is responsible for hemagglutination of red blood cells of different animal species. Type-specific determinants exist on externalized regions of the hexon and on the fiber, which stimulate the neutralizing antibodies responsible for serotype-specific immunity. Similarly, there are type- and genus-specific regions of these genes that are useful targets for diagnostic NATs. Virus DNA replication and assembly of particles take place primarily in the nucleus, although the proteins and capsid aggregates accumulate in the cytoplasm and are often visualized there.

Respiratory adenoviral infections range from sporadic to epidemic; about half are asymptomatic. From 2 to 5% of acute respiratory viral infections in all age groups and up to 18% of such infections in children are due to adenoviruses (Fox et al., 1969; Monto and Sullivan, 1993). The highest figures are observed during epidemics and when NAT or serologic assays are used (Fox et al., 1977; Jennings et al., 2004). By age 10, most individuals have been infected with one or more adenovirus types. Epidemics often occur among children or new military recruits. Other closed, crowded settings can facilitate spread, including day care centers, nursing homes, and hospitals. Intrafamilial infections are also common.

Adenoviral acute respiratory illnesses are usually acquired by contact with contaminated respiratory secretions, stool, or fomites, although airborne transmission also occurs. Spread is efficient due to virus stability. Adenoviruses can remain stable for weeks to months at a variety of temperatures on surfaces and in solution. They are also highly resistant to inactivation by gastric secretions, bile, and pancreatic proteases, so they pass readily through the stomach into the gut. The initial site of infection can be the conjunctivae, oropharynx, or intestine. A brief period of viremia then ensues (Aberle et al., 2003). Virus also spreads to the regional lymphoid tissues where it can replicate for prolonged periods. Children shed the lower-numbered serotypes for up to 3 to 6 weeks in the throat or stool following recovery. Shedding from stool can be prolonged but is not indefinite. Recovery of virus from stool up to 18 months postrecovery has been reported but is uncommon (Brandt et al., 1969; Fox et al., 1977). Viremia is brief but demonstrable in some immune-normal children during illness (Aberle et al., 2003; Shike et al., 2005). Genus- and type-specific IgG antibodies appear 7 to 10 days after onset in older children and adults; neutralizing antibodies may last a decade or more. The IgG response in young children may be delayed and is often directed only at the infecting virus serotype. A rise in titer to heterologous viruses within a genus occurs in about 25% of adults. Virus-specific IgM appears in only 20 to 50% of cases and may reappear upon reinfection.

The incubation period for adenoviral respiratory illness is 2 to 14 days. Common adenovirus-associated URTIs are colds, tonsillitis, pharyngitis, pharyngoconjunctival fever, and occasionally croup. Conjunctivitis accompanies many of these syndromes. Otitis media is a frequent complication in children

TABLE 4 Classification of adenoviruses by species characteristics and involvement in human respiratory disease[a]

Species	Virus type(s)	% G+C in DNA	Hemagglutination		Occurrence of respiratory disease
			Rhesus	Rat	
A	12, 18, 31	47–49	Negative	Negative	Rare
B1	3, 7, 16, 21, 50	50–52	Complete	Negative	Frequent
B2	11, 14, 34, 35	50–52	Complete	Negative	Infrequent
C	1, 2, 5, 6	57–59	Negative	Partial	Frequent
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51	57–60	Negative	Complete	Rare
E	4	58	Negative	Partial	Frequent
F	40, 41	52	Negative	Partial	None

[a]Modified from Robinson and Echevarria, 2007, with permission.

(Chonmaitree et al., 2008). The LRTIs can be severe, prolonged, and cause significant sequellae (Castro-Rodriguez et al., 2006). Syndromes may include tracheobronchitis, bronchiolitis, and an influenza-like illness. A pertussis-like syndrome is described. Adenoviral pneumonia can be fatal, particularly in young children or infants (Abzug and Levin, 1991; Gray et al., 2007). Adenoviruses are a particular problem for SOT or HSCT recipients. The incidence of infections in HSCT patients ranges from 3 to 47%. The source of virus may be a new exposure or reactivation. Adenoviral pneumonia can be an isolated finding or accompany disseminated disease. Mortality is 18 to 52% in HSCT patients and 0 to 35% in SOT recipients, with the highest risk in children (Kim et al., 2007). Virus shedding from the respiratory tract and into stool may be longer than in other groups. Viremia is also prolonged and often detectable before or during disease.

The disease pattern and severity can vary by virus type, by age and immune status of the host, and over time (Table 5). Most adenoviral respiratory illnesses in the developed world are attributable to species B, C, and E. Adenovirus types 1 to 7 cause most respiratory illnesses of children. Types 1, 2, 5, and 6 tend to be endemic, whereas 3, 7, 4, and 14 are often associated with outbreaks or epidemics. A recent U.S. surveillance study identified types 3, 2, 1, 5, 4, and 21, in that order, associated with acute respiratory symptoms in civilians of all ages. Types 5 and 21 appeared to cause the most severe disease (Gray et al., 2007). Simultaneous infections with two or more types can occur in immunocompromised patients, otherwise healthy children, and military recruits (Echavarria et al., 2006; Gray et al., 2007).

Adenoviruses uniquely cause outbreaks of acute respiratory illness associated with considerable morbidity and occasional fatalities in new military recruits. Most prior outbreaks involved serotypes 4 and 7 or serotypes 3 and 21 on occasion, viruses for which most young recruits had no antibody (Ludwig et al., 1998). A live-virus, enteric-coated, oral vaccine against serotypes 4 and 7 was produced and administered to U.S. military personnel from 1971 to 1996 and induced highly effective, local immunity against these viruses (Sanchez et al., 2001). When the vaccine program lapsed, the outbreaks resumed. A replacement vaccine against serotypes 4 and 7 has now been made and is currently undergoing field study. A recent spate of unusually severe LRTIs in recruits due to the rarely reported adenovirus 14, however, may compromise its success (Metzgar et al., 2007). Outbreaks of especially severe pneumonia due to type 14 in otherwise healthy individuals of all ages are also occurring in several regions of the United States, suggesting that a new and virulent variant of type 14 variant has emerged (Centers for Disease Control and Prevention, 2007).

During acute infection, adenovirus can be detected in respiratory secretions or stool by tube culture of epithelial cells such as A549, Hep-2, or HeLa. Growth usually appears in 2 to 5 days but can take as long as 10 to 14 days. Virus can be presumptively identified by its characteristic CPE of grape-like clusters. Confirmation is recommended and is most often performed by immunologic methods such as IF or EIA, using antibodies against the common region of the hexon. Centrifugation cultures reduce time to detection, but sensitivity compared to tube culture is lower (50 to 85%) than for most other respiratory viruses, especially when mixed cell cultures are used (Espy et al., 1987; LaSala et al., 2007). Serology and molecular assays detect more adenovirus infections than culture (Fox et al., 1977; Jennings et al., 2004). Adenovirus serology is useful for epidemiologic studies but is impractical for clinical use because acute- and convalescent-phase specimens are required and results may be unreliable in immunocompromised patients. Classic methods to serotype isolates include neutralization or inhibition of hemagglutination (Robinson and Echavarria, 2007). Molecular typing methods are increasingly used, are rapid, and compare favorably to the classic methods (Sarantis et al., 2004; Gray et al., 2007).

Rapid diagnosis of adenoviral infections is most often performed by detection of viral antigen. The most common approach is IF, although an immunochromatography kit is commercially available (Fujimoto et al., 2004). Sensitivity of IF is lower for adenovirus than for other respiratory viruses (40 to 60% compared to culture) (Landry and Ferguson, 2000). Molecular assays are increasingly used for rapid detection of adenovirus in respiratory specimens (Pehler-Harrington et al., 2004; Echavarria et al., 2006; Nolte et al., 2007). The multiplex NAT recently approved for diagnostic use in the United States, however, may underdetect some adenovirus types (Pabbaraju et al., 2008). In most studies, NAT had modestly increased adenovirus yield from respiratory specimens compared to culture (Tsolia et al., 2004; Richard et al., 2008). Coinfections involving adenoviruses are frequently identified by NATs (Jennings et al., 2004; Weigl et al., 2007). Adenoviral DNA can be detected in serum samples of otherwise-healthy children and immunocompromised patients during acute respiratory episodes (Echavarria et al., 2001; Aberle et al., 2003). Viral load assays are especially useful for early detection and monitoring of the prolonged

TABLE 5 Common adenovirus serotypes associated with respiratory tract diseases in different populations

Disease(s)	Associated virus type(s)[a]		Usual hosts
	Frequent	Infrequent	
URTI	1–3, 5, 7	4, 6, 11, 14b, 15, 18, 21, 29, 31	Infants, children
Pneumonia, other LRTIs	3, 4, 7, 21	1, 2, 5, 7, 8, 11, 14, 35	Infants, children, immunocompromised individuals
Acute respiratory tract disease	4, 7	2, 3, 5, 8, 11, 14, 21, 35	Military recruits
Pertussis syndrome	5	1, 2, 3, 12, 19	Children
Pharyngoconjunctival fever	3, 4, 7	1, 2, 5, 6, 8, 11–17, 19–21, 29, 37	Children
Acute conjunctivitis	1–4, 7	6, 9, 10, 11, 15–17, 19, 20, 22, 37	Children
Epidemic keratoconjunctivitis	8, 9, 37	2, 3, 4, 5, 7, 10, 11, 13–17, 19, 21, 23, 29	Individuals of any age

[a]Other adenovirus types may be associated with these syndromes infrequently or sporadically. Modified from Robinson and Echavarria, 2007.

viremia that occurs in transplant patients with adenovirus-associated diseases, including pneumonia (Leruez-Ville et al., 2004). Quantitative NAT of respiratory specimens may have prognostic value, although further studies of this approach are needed (Faix et al., 2004; Shike et al., 2005).

A positive adenovirus result in the respiratory tract may be difficult to interpret due to persistent virus shedding. Indeed, up to 25% of asymptomatic individuals can shed virus, depending on the patient population, specimen type, and assay (Brandt et al., 1969; Echavarria et al., 2003; Faden et al., 2005; van Gageldonk-Lafeber et al., 2005). Lesser amounts of virus, however, appear to be shed by asymptomatic individuals than by ill individuals. For example, a thousand copies of adenovirus per microgram of DNA were detected in one study of bronchoalveolar lavage fluids from asymptomatic adults, whereas three logarithms more was found in respiratory swabs from symptomatic children (Leung et al., 2005a; Shike et al., 2005). Identification of adenovirus in respiratory specimens, particularly by less-sensitive methods such as IF, is usually considered presumptive evidence of infection. Higher values in quantitative assays may likewise be significant.

Management of adenoviral infections is largely supportive. A number of antiviral drugs, the nucleoside analogues ribavirin, vidarabine, and cidofovir, have been used for treatment. Cidofovir has shown the most promising efficacy for treatment of adenovirus LRTI in high-risk groups (Leruez-Ville et al., 2004; Doan et al., 2007; Anderson et al., 2008). The redeveloped vaccine against adenoviruses 4 and 7 will be used for military personnel only.

Bocavirus

In 2005, a large-scale molecular screening program designed to detect new viruses identified a previously undescribed parvovirus in 3.1% of respiratory secretions from Swedish children with respiratory illnesses. The newly identified virus was named human bocavirus (HBoV) based on its phylogenetic relationship to the *ca*nine minute virus and the *bo*vine parvovirus, the only other members of a recently defined *Bocavirus* genus of the *Parvoviridae* family (Table 1) (Allander et al., 2005). This discovery created considerable interest because HBoV is only the second parvovirus with possible pathogenicity for humans. Other human parvoviruses are B19 which causes the rash illness called Fifth disease and other nonrespiratory syndromes, the presumably apathogenic adenovirus-associated viruses, and PAR4, which was recently identified in blood but has not been linked to human disease.

HBoV has now been cloned and completely sequenced (Allander et al., 2005). It has not been grown in cell culture or visualized by electron microscopy, nor is there an animal model. Therefore, most studies have relied on its identification by NAT. Like other members of the *Bocavirus* genus, the HBoV virion is nonenveloped and contains a linear, single-stranded DNA molecule of about 5 kb. The genome encodes at least two capsid proteins, VP1 and VP2, and two nonstructural proteins, NS1 and NP2. Each of these proteins is clearly distinct from their canine and bovine homologues (Allander et al., 2005). The most conserved regions are in the NS1 and NP1 genes. Greater variation is observed in the capsid genes, giving rise to at least two clusters of HBoV. Differences between these clusters are small, suggesting a single HBoV lineage with 2 genotypes with subtle but distinct, globally conserved variations (Kesebir et al., 2006; Bastien et al., 2007; Chieochansin et al., 2007; Neske et al., 2007).

HBoV has now been identified in 1.5 to 13.9% of respiratory tract specimens submitted to diagnostic laboratories in North America, Europe, Asia, and Australia, suggesting worldwide circulation. It is identified less often than RSV and rhinoviruses, is approximately as common as HMPV, parainfluenza virus type 3, and the adenoviruses, and is seen more frequently that the CoVs and the other parainfluenza viruses. Serosurveys from several countries using either baculovirus-expressed VP1 or virus-like particles generated from VP2 suggest that HBoV infections are common and acquired by over 70% of children by 4 to 6 years of age (Endo et al., 2007; Kahn et al., 2008). Peak incidences seem to be during the cooler months of the year.

Most HBoV-positive specimens are obtained from otherwise healthy infants and children under 3 years of age with acute respiratory distress (Kesebir et al., 2006; Manning et al., 2006; Calvo et al., 2008; Smuts et al., 2008). Both URTIs and LRTIs are reported. The most frequent signs and symptoms among hospitalized children include fever, cough, rhinorrhea, bronchiolitis, and croup. A significant association with expiratory wheezing was noted by several groups (Allander et al., 2005; Kesebir et al., 2006; Calvo et al., 2008; Smuts et al., 2008). In one such study, HBoV was found in 19% of wheezing children with asthma and was the only viral pathogen identified in 5% (Allander et al., 2007b). Also noted is a paroxysmal cough clinically resembling that induced by *Bordetella pertussis* (Arnold et al., 2006). Studies of immunocompromised patients are limited so far, but severe pneumonia occurred in an adult with lymphoma. Virus was also persistently shed or reactivated concurrently with fevers over a 6-month period by 3 children with acute lymphoblastic leukemia (Kupfer et al., 2006; Koskenvuo et al., 2007b). Overall, no clinical features clearly distinguish HBoV-associated respiratory illnesses from those induced by other respiratory viruses.

The ability of HBoV to cause acute respiratory illness, however, is not clear. One reason is that an extremely high coinfection rate is observed. On average, 40% of specimens containing HBoV also contain other respiratory viruses, with values from 25% to over 80% reported. The highest coinfection rates are observed when more viral targets are sought and NAT is employed (Allander et al., 2005; Allander et al., 2007b; Fry et al., 2007). Although HBoV can be codetected with any respiratory virus, a striking 69% of specimens containing HBoV also contained adenovirus in one study (Hindiyeh et al., 2008). Another reason for uncertainty is that variable results are reported in controlled trials. One study demonstrated a significant association of the virus with LRTI when outpatients with influenza-like illness were compared to hospitalized individuals with pneumonia (Fry et al., 2007). Another investigation identified HBoV in 5.2% of various respiratory specimens from 425 symptomatic U.S. children less than 2 years of age with acute respiratory symptoms but in none of 96 nasal washes of asymptomatic children (Kesebir et al., 2006). Only 5% of asymptomatic Spanish children shed HBoV, compared to 17% of ill hospitalized subjects (Garcia-Garcia et al., 2008). Yet a recent study of Danish infants found HBoV in similar proportions (8.2% compared to 8.6%) of nasal swabs from case and control subjects (von Linstow et al., 2008).

Quantification of virus has been suggested to discriminate HBoV infection from disease. Despite multiple attempts, no consistent pattern has emerged, perhaps due to the challenge of collecting and testing respiratory specimens in a standardized fashion. Most studies find that HBoV loads in symptomatic individuals vary considerably, from 100 to 10^{10} copies/ml of secretions. Low to moderate viral loads (less than 1×10^4 copies/ml) are most common. There is also considerable

overlap in viral loads of ill and asymptomatic subject groups (Allander et al., 2007b; Kleines et al., 2007; Neske et al., 2007). In conclusion, HBoV appears to be associated with respiratory tract illness, particularly in children. Yet its significance as a primary pathogen, cofactor, or innocent bystander remains to be determined.

HBoV has also been identified in serum of children with respiratory tract symptoms, particularly those with high viral loads in the respiratory tract. This finding suggests that the virus may also cause disease beyond the respiratory tract (Allander et al., 2007b; Fry et al., 2007). Indeed, an association between HBoV and Kawasaki disease has been proposed (Catalano-Pons et al., 2007). The virus has also been detected in the feces of children with and without respiratory tract symptoms, some of whom also had diarrhea (Lau et al., 2007; Lee et al., 2007; Vicente et al., 2007). Similarly, some animal bocavirus infections also cause respiratory and gastrointestinal infections. It has been proposed that detection of HBoV in the human gastrointestinal tract may be incidental, perhaps due to swallowing of the virus during respiratory infection, to reactivation by other enteric pathogens, or to coinfection. The ingestion hypothesis is supported by the fact that patients with detectable HBoV in stool tend to be those with the highest viral loads in the respiratory tract (Neske et al., 2007). Coinfections with HBoV and other established enteric pathogens are almost as frequent, up to 56% in one recent study, as are coinfections in the respiratory tract (Lau et al., 2007). Identification of HBoV in the same proportion (3.5%) of stools from children with acute gastroenteritis and a known enteric virus and in asymptomatic children now casts doubt on HBoV as a primary agent of gastroenteritis (Cheng et al., 2008). Thus, the relationship between HBoV, diarrhea, and respiratory disease also remains to be clarified.

Polyomavirus

Novel viruses in the *Polyomvaviridae* family have recently been linked to human pulmonary disease as well (Table 1). In early 2007, two research groups independently reported polyomaviruses in respiratory samples submitted to clinical laboratories, using molecular pathogen discovery methods similar to those that identified HBoV. The polyomavirus identified at the Karolinska Institute in Stockholm, Sweden was initially found in pools of banked nasopharyngeal aspirates submitted to a diagnostic laboratory and was given the name KI (Allander et al., 2005). A month later, another polyomavirus was reported in a nasopharyngeal aspirate of a 3-year-old Australian child with pneumonia by a group at Washington University School of Medicine in the United States. It was present in many respiratory samples from U.S. and Australian cohorts and was called WU (Gaynor et al., 2007). KI has now been found in feces, whole-blood, leukocyte, and serum samples, but neither virus appears to be detected in urine.

WU and KI, like other primate-infecting members of the *Polyomaviridae*, are small, icosahedral, nonenveloped viruses containing closed circular double-stranded DNA of 4 to 5 kb. Their genome contains an early region encoding two regulatory proteins known as small t (tumor) and large T antigen and a late region which specifies the viral capsid proteins VP1, VP2, and VP3. Phylogenetic analysis demonstrates considerable homology between the early proteins of WU and KI and other primate polyomaviruses, but late proteins are highly divergent in sequence and size. Thus WU and KI may form a new branch within the *Polyomaviridae*. Multiple alignments of WU and KI sequences indicate that WU is closely related but not identical to KI and that strains of each virus may exist. The finding of new polyomaviruses in the human respiratory tract has generated considerable interest because, previously, the only known human polyomavirus pathogens were JC and BK, which cause widespread, persistent, but mostly asymptomatic, infections in immune-normal individuals. In the context of immunosuppression, however, JC can cause progressive multifocal leukoencephalopathy and BK is associated with renal and urinary tract disorders. Polyomaviruses of animals other than humans have oncogenic potential.

WU and KI have now been detected in a variety of upper and lower respiratory tract specimens across four continents, suggestive of a global distribution. Prevalence ranges from 0.4 to 7% for WU and 0.6 to 2.6% for KI (Allander et al., 2007a; Gaynor et al., 2007; Lin et al., 2008). Both viruses appear to circulate year-round, although WU may be found more in winter to spring (Norja et al., 2007; Bialasiewicz et al., 2008). They have been identified in all age groups, but the highest detection rates are in children less than 3 years old. Interestingly, many virus-positive older children and adults have altered immune status. This finding suggests that persistent or latent infections may be established early in life and perhaps accelerate or reactivate when immunity is compromised (Gaynor et al., 2007; Le et al., 2007; Norja et al., 2007). Indeed, WU may persist for 6 to 8 weeks and perhaps longer in immunocompromised patients (Le et al., 2007). The most common clinical manifestations in the majority of symptomatic subjects are URTIs with fever and cough. Bronchiolitis and pneumonia have also been described. Vertical transmission from mother to fetus was suggested in one case (Allander et al., 2007a; Le et al., 2007).

The importance of WU and KI as respiratory pathogens, like that of HBoV, remains unclear. Certainly the clinical manifestations of the respiratory polyomaviruses are not unique among respiratory viruses. In addition, other respiratory viruses are often identified in positive specimens. Indeed, codetection rates as high as 31 to 79% for WU and 33 to 74% for KI have been reported (Le et al., 2007; Norja et al., 2007; Lin et al., 2008). Few statistically significant differences in symptoms occur between patients infected with WU or KI alone and individuals coinfected with other respiratory viruses (Le et al., 2007). There is also considerable overlap in virus detection in cases and controls. In three such studies, prevalence of WU or KI in symptomatic patients ranged from 1 to 7% compared to 0 to 6.4% in asymptomatic subjects (Abed et al., 2007; Han et al., 2007; Norja et al., 2007). The ability of these polyomaviruses to persist for prolonged periods in immunocompromised subjects and the high proportion of such individuals in many reported studies also complicates assessment of their significance. Additional studies are needed to clarify their function as pathogens or passengers in acute human respiratory disease.

CONCLUSIONS

The respiratory virus field has recently been energized by significant changes. The number of real and potential respiratory viruses has increased considerably, and improved laboratory methods to detect them now abound. These advances have greatly improved and, in many cases, substantially altered our understanding of the epidemiology and pathogenesis of respiratory virus disease. A specific, rapid diagnosis in the hospital or emergency room setting can improve outcomes such as decreased antibiotic and ancillary test use, particularly for pediatric populations. The value of rapidly identifying

and limiting the global spread of SARS-CoV is undisputed. Similarly, close monitoring of the continued incursions of highly pathogenic AI A viruses into the human population may help prevent the next pandemic.

Yet many challenges remain. The nonspecific nature of acute respiratory virus illnesses and the considerable overlap of their signs and symptoms render clinical diagnoses unreliable in most circumstances. It is also unclear if several of the newly discovered respiratory viruses cause or even contribute to disease. The paucity of vaccines and antiviral therapy for viruses other than influenza is especially concerning, with even established antivirals now ineffective against some currently circulating influenza virus strains. An added concern is that most ill individuals present to medical attention when viral loads are declining, so the efficacy of current antivirals is usually modest. The enormous medical and economic burden that respiratory virus infections create will no doubt increase in upcoming years as the proportion of elderly and other high-risk groups in the population expand worldwide. Globalization likewise adds to the potential for animal respiratory viruses to encounter susceptible human populations, adapt, and spread. Therefore, continued development of methods to rapidly diagnose relevant respiratory virus infections, to prevent infection, and to limit disease severity remain essential goals.

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Enteroviruses and Parechoviruses

MARK A. PALLANSCH AND M. STEVEN OBERSTE

18

INTRODUCTION

Human enteroviruses (EV) are members of the *Enterovirus* genus of the family *Picornaviridae* and are among the most common human viral infections. Although most EV infections are asymptomatic, millions of symptomatic EV infections are estimated to occur each year in the United States (Khetsuriani et al., 2006; Pallansch and Roos, 2006). Most EV infections cause only mild nonspecific disease, but infections can infrequently lead to serious illness and hospitalization, especially in infants and in those who are immunecompromised. In addition, this group of viruses is the most common cause of aseptic meningitis, the most frequent central nervous system (CNS) infection (Khetsuriani et al., 2003). EV have also been implicated in other acute and chronic diseases. Two former EV, now recognized as members of a new virus genus in the family *Picornaviridae*, *Parechovirus*, also have a somewhat similar clinical spectrum and physical properties, but they are genetically distinct and present new diagnostic challenges.

History of Virus Discovery

The history of EV begins with the history of poliovirus (PV). Many of the early PV studies are landmarks in the study of EV and all of virology. The first clinical descriptions of poliomyelitis were made in the 1800s, with reports of cases of paralysis with fever. Understanding of the infectious nature of this disease began with studies in the early 20th century. These studies recognized the communicable nature of poliomyelitis, the importance of asymptomatic infection in the transmission of PV, and the role of enteric infection in disease pathogenesis. In a classic study, Viennese investigators Landsteiner and Popper proved the infectious nature of poliomyelitis by inoculation of CNS tissue homogenates from human cases into monkeys and successfully transmitting the clinical disease and its pathology to a nonhuman host (Landsteiner and Popper, 1908).

Building on studies of others, Enders et al. showed that PV could be propagated in nonneural tissue culture (Enders et al., 1949). These investigations had implications for all of virology because they indicated, first, that PV grew in various tissue culture cells that did not correspond to the tissues infected during the human disease and, second, that PV destroyed cells with a specific cytopathic effect (CPE). Neutralization tests showed that PV has three serotypes (Bodian et al., 1949), and serologic tests (Aycock, 1928) confirmed that most infected individuals do not manifest clinical disease. These investigations laid a critical framework for the development of a vaccine, and they clarified much confusing earlier data, such as the apparent occurrence of second attacks of poliomyelitis.

The control of polio began with the production of two different vaccines: the Salk inactivated polio vaccine (IPV) delivered via intramuscular injection (licensed in 1955 in the United States) and the orally delivered Sabin live, attenuated vaccine (oral polio vaccine [OPV], licensed in 1961–1962). PV studies have continued as a model virology system and continue to have a significant impact on the field of molecular virology. PV was the first animal virus completely cloned and sequenced (Kitamura et al., 1981; Racaniello and Baltimore, 1981b), the first infectious clone for an RNA animal virus (Racaniello and Baltimore, 1981a), and the first human virus whose three-dimensional structure was solved by X-ray crystallography (Hogle, 1982; Hogle et al., 1985). In 1989, Mendelson et al. (Mendelsohn et al., 1986; Mendelsohn et al., 1989) identified the PV receptor, CD155, a finding that was followed by the generation of transgenic mice carrying the human PV receptor (Horie et al., 1994; Ren and Racaniello, 1992; Ren et al., 1990).

Coxsackieviruses (CV) were first isolated from the feces of paralyzed children following a poliomyelitis outbreak in 1947 in Coxsackie, New York (Dalldorf and Sickles, 1948). These isolates were detected by observing paralysis following intracerebral inoculation of suckling mice. In the following year, the first member of the CV group B (CVB) was isolated from cases of aseptic meningitis (Melnick et al., 1949). The original CV group A (CVA) isolates produced myositis with flaccid hind limb paralysis in newborn mice, whereas the CVB produced a spastic paralysis and generalized infection in newborn mice, with myositis as well as involvement of the brain, pancreas, heart, and brown fat (Dalldorf, 1950; Gifford and Dalldorf, 1951).

In 1951, echoviruses were first isolated in tissue culture from the stools of asymptomatic individuals (Melnick and Ågren, 1952). Echoviruses received their name because they were *e*nteric isolates, *c*ytopathogenic in tissue culture, isolated from *h*umans, and *o*rphans (i.e., not associated with a known clinical disease). Subsequent studies have shown that echoviruses, in fact, do cause a variety of human diseases (Kibrick et al., 1957; Melnick, 1954; Riordan et al., 1952; Robbins et al., 1951; Stobo et al., 1974; Weller, 1952; Weller et al., 1952).

Virus Classification

Historically, the classification of EV into the subgroups of PV, CVA, CVB, and echoviruses was based on the empirical observations of their association with certain clinical syndromes or disease, tissue tropism, nature of disease in suckling mice, growth in certain specific cell cultures, and in some cases, antigenic similarities (Committee on Enteroviruses, 1962; Committee on the ECHO viruses, 1955; Committee on the Enteroviruses, 1957; Panel for Picornaviruses, 1963). Using these criteria, 67 different serotypes were recognized and classified, despite the fact that several antigenically related viruses had different pathogenic properties in mice (Panel for Picornaviruses, 1963). This last discrepancy eventually led to the dropping of the old designations; thereafter, new serotypes were simply termed "enterovirus" followed by a number, beginning with EV68 (Melnick et al., 1974; Schieble et al., 1967). A more satisfactory, and completely new, classification system followed from the advent of comprehensive genetic studies. The complete RNA genomic sequences for all the recognized EV have been determined, which has allowed a more detailed description and comparison among these viruses (Andersson et al., 2002; Blackburn et al., 1992; Brown et al., 2003; Brown and Pallansch, 1995; Chang et al., 1989; Dahllund et al., 1995; Earle et al., 1988; Hughes et al., 1989; Iizuka et al., 1987; Inoue et al., 1989; Jenkins et al., 1987; Junttila et al., 2007; Klump et al., 1990; Kraus et al., 1995; Lindberg et al., 1987; Lindberg et al., 1999; Lindberg and Polacek, 2000; Martino et al., 1999; Newcombe et al., 2003; Nomoto et al., 1982; Norder et al., 2003; Oberste et al., 1998, 2004a, 2007b; Oberste et al., 2004c; Oberste et al., 2004d; Oberste et al., 2005; Oberste et al., 2007a; Ohman et al., 2001; Polacek et al., 1999; Pöyry et al., 1994; Ryan et al., 1990; Stanway et al., 1984; Supanaranond et al., 1992; Toyoda et al., 1984; Zhang et al., 1993; Zimmermann et al., 1995; Zimmermann et al., 1996). From these new data, and to avoid the inconsistencies of the previous classification scheme, the human EV have been reclassified into five species: *Human enterovirus* A to *D* and *Poliovirus* (Stanway et al., 2005). More-recent characterization of previously unidentified EV and an increased emphasis on genetic relationships rather than clinical disease has resulted in the proposal that the three PV be reclassified as members of *Human enterovirus* C (http://www.picornastudygroup.com/proposals/2007/proposals_2007.htm). The new classification for picornaviruses affecting humans is given in Table 1.

Continued molecular characterization of EV clinical isolates has also identified many new members of the genus, with the naming continuing sequentially from EV73 and extending up to at least the proposed EV103 (Junttila et al., 2007; Norder et al., 2003; Oberste et al., 2001; Oberste et al., 2004c; Oberste and Pallansch, 2005; Oberste et al., 2007a). These have been assigned to one of the four species and are included in Table 1 for reference. At this time, very little is known about any distinctive clinical or epidemiologic features of these new viruses, but it is clear that there are likely to be many more of these described with the wider application of sequencing studies to viruses from the developing world. Based upon similar genetic comparisons, there is also a proposal to reclassify the two human *Rhinovirus* species as members of the *Enterovirus* genus (Table 1).

Molecular studies also have demonstrated that echovirus 22 (E22) and E23 are genetically distinct from the EV (Coller et al., 1990; Hyypiä et al., 1992; Stanway et al., 1994). On the basis of a very low genetic relationship, differences in viral proteins and processing, and a novel protease, E22 and E23 were reclassified as members of the new picornavirus genus *Parechovirus* and renamed human parechoviruses 1 and 2, respectively (Stanway et al., 2000; Stanway et al., 2005). Additional members of this genus have been recently identified, including both additional serotypes of human parechovirus (Al-Sunaidi et al., 2007; Benschop et al., 2006a; Ito et al., 2004; Watanabe et al., 2007b) as well as a separate species first isolated in Swedish bank voles, Ljungan virus (Niklasson et al., 1999). Ljungan virus has been associated with diabetes in its natural host and may have a possible role in human disease (Niklasson et al., 1998; Niklasson et al., 2003a; Niklasson et al., 2003b; Niklasson et al., 2006a; Niklasson et al., 2006b; Niklasson et al., 2007). Another distinct

TABLE 1 Picornavirus genera, species,[a] and serotypes affecting humans

Genus and species	No. of serotypes	Serotype(s)
Genus *Enterovirus*		
Human enterovirus A	17	CVA2–8, 10, 12, 14, 16 ; EV71, 76, 89–92
Human enterovirus B	56	CVA9; CVB1–6; E1–7, 9, 11–21, 24–27, 29–33; EV69, 73–75, 77–88, 93, 97–98, 100, 101
Human enterovirus C	16	CVA1, 11, 13, 17, 19–22, 24; PV1–3; EV95–96, 99, 102
Human enterovirus D	3	EV68, 70, 94
Human rhinovirus A	75	HRV1–2, 7–13, 15–16, 18–25, 28–34, 36, 38–41, 43–47, 49–51, 53–68, 71, 73–78, 80–82, 85, 88–90, 94–96, 98, 100, Hanks
Human rhinovirus B	25	HRV3–6, 14, 17, 26–27, 35, 37, 42, 48, 52, 69–70, 72, 79, 83–84, 86, 91–93, 97, 99
Genus *Hepatovirus*	1	Hepatitis A virus
Genus *Cardiovirus*		
Encephalomyocarditis virus	1	Encephalomyocarditis virus
Theilovirus	2	Saffold virus 1–2
Genus *Kobuvirus*[b]	1	Aichi virus
Genus *Parechovirus*	6	HPeV1–6

[a]The classification scheme shown is adapted from the Picornavirus Study Group of the International Committee for the Taxonomy of Viruses (http://www.picornastudygroup.com/) (Stanway et al., 2005) and divides serotypes into different species based on genome organization, sequence similarity, and other physical properties. Many of the listed species and genera also contain members not listed that affect mammalian hosts other than humans and that, to date, have no documented role in human disease.

[b]Proposed as a genus.

picornavirus genus associated with human infection is *Kobuvirus* (Yamashita et al., 1998). Although little information is currently available about this virus, it appears that it is often associated with gastroenteritis in young children and infection is common. What is notable about both of these newer genera is that the currently available molecular reagents for the detection of EV do not detect these viruses (see "Diagnosis").

BIOLOGY

Structure

EV are small RNA viruses consisting of a spherical, nonenveloped capsid that contains a single-stranded positive-sense RNA genome (Fig. 1). Many of the structural and functional properties of EV and parechoviruses are conserved and collectively distinguish these genera from other genera in *Picornaviridae* (Stanway et al., 2005).

The typical EV particle has a diameter of 30 nm, a buoyant density of 1.30 to 1.34 g/ml in CsCl gradients, a molecular mass of 8.6×10^6 Da, and a sedimentation coefficient of 156S to 160S (Racaniello, 2007). The icosahedral virus capsid, consisting of 60 protomers, is assembled from 12 identical pentamers. Each protomer is composed of the four virus proteins, VP1, VP2, VP3, and VP4. VP1, VP2, and VP3 constitute most of the capsid surface, whereas VP4 is located in the interior of the capsid in close contact with the viral RNA (Lentz et al., 1997). In the case of parechoviruses, the protomer consists of only three proteins, VP0, VP1, and VP3, since VP0 does not undergo the maturation cleavage to VP4 and VP2 that occurs in the EV (Stanway and Hyypiä, 1999; Stanway et al., 2000). One of the major structural features of the capsid surface is a depression, called the "canyon," around each of the 12 fivefold symmetry axes. The canyon is formed at the junction of VP1 and VP2-VP3 and is involved in the binding of the virus-specific receptor (Rossmann, 1989a, 1989b). A sphingosine-like molecule binds in a pocket formed by VP1 at the bottom of the canyon (Filman et al., 1989).

The genomes of the human EV are approximately 7,500 nucleotides long and encode a single open reading frame for all viral capsid and functional proteins (Fig. 2). The genomes of almost all EV types have been sequenced completely (for updated information, see "Picornavirus Home" [http://www.picornaviridae.com/]). A small virus protein (VPg, for "virus protein—genome-linked") is covalently bound to the 5′ end of the RNA molecule, and the 3′ end of the virion RNA is polyadenylated. The 5′ and 3′ termini of the viral RNA include nontranslated regions (NTRs) of different lengths (5′ NTR, 711 to 755 nucleotides; 3′ NTR, 69 to 109 nucleotides), each showing a high degree of secondary structure. Two major secondary structures in the 5′ NTR serve as an internal ribosomal entry site (IRES) for the initiation of viral protein synthesis at the correct AUG codon (position 743 in PV type 1, Mahoney strain) and a cloverleaf structure involved in RNA replication (Barton et al., 2001; Herold and Andino, 2001).

The positive-sense viral RNA serves as mRNA. The translation of this RNA yields a single large precursor polyprotein, consisting of three major functional domains (Fig. 2). Region P1 codes for the capsid proteins VP0 (precursor of VP4 and VP2), VP3, and VP1. Regions P2 and P3 encode functional proteins such as the viral proteases (2A, 3C, and 3CD), the genome-linked virus protein VPg, the viral RNA polymerase (3D), and other accessory proteins. The P1 capsid precursor protein is released from the nascent polyprotein by intramolecular protease 2A cleavage. In additional steps, P1 and the precursor for the functional proteins (P2 and P3) are processed by proteases 3C and 3CD. The final cleavage of VP0 into VP4 and VP2 takes place when the newly synthesized viral RNA is encapsidated at the end of the virus maturation process. Within the infected cell, the replication of the viral RNA has been studied intensively, and much has been learned in recent years about the steps in the replication process (Fig. 3). Excellent reviews of these details at the molecular level have been published (Racaniello, 2007, and references therein).

Antigenicity and Neutralization

The picornaviruses are among the simplest RNA viruses, having a highly structured capsid with limited surface elaboration. Yet, despite the limited genetic material and structural constraints, evolution within the picornaviruses has resulted in a large number of readily distinguishable members. This variability has been categorized antigenically as serotype. Each of the serotypes correlates with the immunologic response of the human host, protection from disease, receptor usage, and to a lesser extent, the spectrum of clinical disease. These correlations, however, have only a partial relationship with the original classification of EV into PV, CVA or CVB, and echoviruses. Within each of these groups, isolates can usually be readily distinguished on the basis of antigenicity, as measured with antisera raised in animals (Beeman et al., 1952; Contreras et al., 1952; Dalldorf and Sickles, 1956). These antigenic groupings, which define the serotypes, became increasingly more complicated as the number of different viruses grew (Committee on Enteroviruses, 1962). The distinction between serotypes also began to blur as new isolates were discovered that bore only weak antigenic relationships to known serotypes.

The molecular studies also have provided a framework in which the EV antigenic relationships can be better understood. These studies suggest that the nucleotide sequence of VP1 is an excellent surrogate for antigenic typing by means of neutralization tests to distinguish EV serotypes (Caro et al., 2001; Casas et al., 2001; Norder et al., 2001; Oberste et al., 1999b; Oberste et al., 1999c) . In addition to the genetic relatedness, many different EV serotypes share some antigenicity. For example, PV1 and PV2 share a common antigen, and antigenic relationships also exist between CVA3 and CVA8, CVA16 and EV71, E6 and E30, and E12 and E29. When virions are disrupted by heating, particularly in the presence of detergent, nonsurface antigens are exposed that are shared broadly among many EV (Mertens et al., 1983). Despite these limitations, the serotype remains the single most important physical and immunologic property that distinguishes the different EV.

In addition to the 90 or more serotype prototype strains, antigenic variants exist for several EV. One type of antigenic variant is the prime strain, identified for many EV, including CVA17 and CVA24; E1 to E6, E9, E11, E29, and E30; and EV70 (Abraham, 1967; Jackson and Muldoon, 1973; Schmidt et al., 1966). A prime strain is defined by the following properties relative to the prototype strain (Schmidt et al., 1966): reference antisera to the prototype strain neutralize the prime strain poorly or not at all, whereas antisera prepared to the prime strain neutralize the prime strain and the prototype strain equally well. A prime strain, thus, appears to elicit a broader spectrum of neutralizing antibodies, suggesting the presence of additional epitopes compared to the corresponding prototype strain.

A second type of antigenic variant, referred to as an intratypic variant, also has been identified for some EV, including

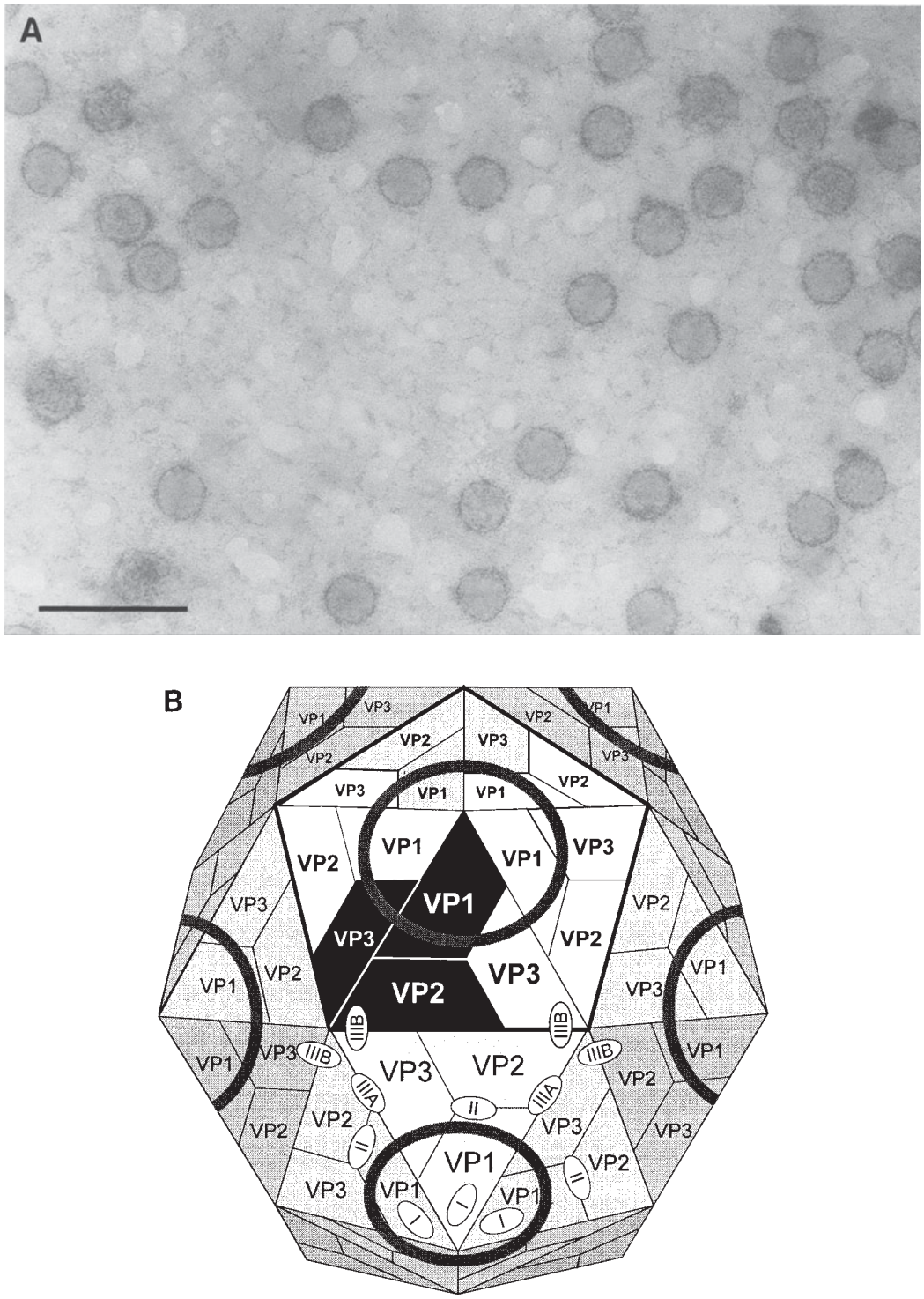

FIGURE 1 Morphology of PV. (A) Transmission electron micrograph of PV type 1 particles, negatively stained with 0.5% uranyl acetate. Bar, 100 nm. (B) Schematic representation of the three-dimensional structure of a PV particle and the four neutralizing antigenic (N-Ag) sites. X-ray crystallographic structure analysis of PV type 1 (Hogle et al., 1985) has revealed an icosahedral capsid structure typical of EV. The capsid surface is composed of 60 protomers, each consisting of the capsid proteins VP1, VP2, and VP3 (black areas). Each of the 12 fivefold symmetry axes is surrounded by five protomers, forming a pentamer (surrounded by a bold black line). The attachment site for the virus-specific receptor is a depression around the fivefold symmetry axis, also called the canyon (dark gray circles). Each of the three surface-exposed capsid proteins contains immunodominant antigenic sites at which neutralizing antibodies bind, resulting in neutralization of virus infectivity. Four N-Ag sites (white ellipses) have been mapped to surface loop extensions (for a review, see Hogle and Filman, 1989). N-Ag I is a continuous sequence in VP1 mapping to amino acids 95 to 105. N-Ag II is a discontinuous site mapping to amino acids 221 to 226 of VP1 and amino acids 164 to 172 and 270 in VP2. N-Ag III consists of two independent discontinuous sites. N-Ag IIIA is composed of amino acids 58 to 60 and 71 to 73 of VP3, and N-Ag IIIB consists of amino acid 72 of VP2 and amino acids 76 to 79 of VP3. The smallest capsid protein, VP4, lies buried in the capsid shell in close association with the single molecule of viral RNA.

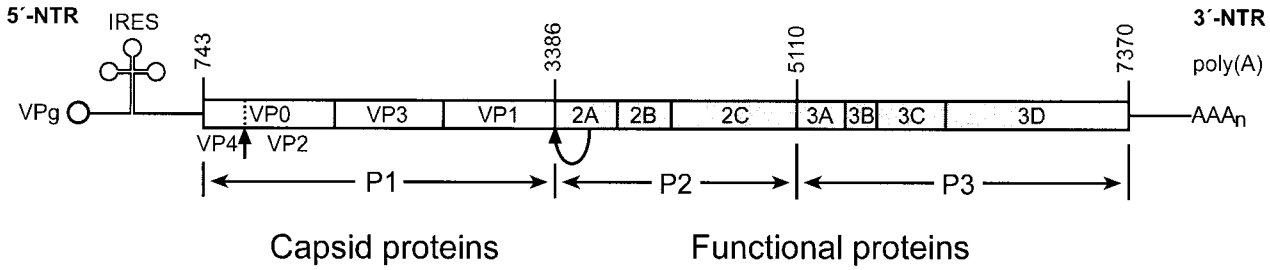

FIGURE 2 Genome organization of PV type 1. The PV genome is a single-stranded positive-sense RNA of approximately 7,500 nucleotides. Nucleotides 743 to 7370 encode in a single open reading frame the capsid proteins (white boxes in coding regions P1) and functional proteins (gray boxes in coding regions P2 and P3). The 5′ and 3′ NTRs are shown as lines. The IRES is shown schematically with the two-dimensional structure. The virus protein VPg is covalently linked to the terminal uracil of the 5′ NTR. For further details, see "Structure" and "Replication in Cell Culture" in the text.

CVA24, CVB1 to CVB4, CVB6, E4, E9, E33, and EV70 (Abraham and Inverso, 1977; Margalith et al., 1968; Schmidt and Lennette, 1970; Subrahmanyan et al., 1973). Such variants generally have an antigenic spectrum that differs from (possibly narrower and overlapping) their corresponding prototype strains and thus cannot be classified as prime strains. The reference antisera and antisera prepared against the intratypic variant have a reciprocal, reduced neutralization activity with the heterologous strain compared with the homologous one. The origin of prime and intratypic variant strains is speculative, but they may result from either antigenic drift or recombination. The specific epitope changes among these antigenic variants have not been described, and therefore, the structural changes involved are not known. In addition, it is not understood why some serotypes have numerous prime strains, others have antigenic variants, and still others are antigenically homogeneous.

Despite this lack of understanding of molecular variation in virus structure as measured by polyclonal antibodies, high-resolution studies of the virion surface have been particularly useful in identifying the targets of neutralization of EV by monoclonal antibodies (Mateu, 1995; Minor, 1986). EV isolates are routinely characterized by serotype using polyclonal animal sera, and by this criterion, the serotypes appear to be stable and well defined. Studies using monoclonal antibodies prepared against CVB3 and CVB4 suggest, however, that considerable antigenic variation exists within a CVB serotype (Prabhakar et al., 1987; Reimann et al., 1991). Monoclonal antibodies have been used to study CVB strains, plaque variants of the prototypes, and clinical isolates (Prabhakar et al., 1982; Prabhakar et al., 1985). In all cases, multiple differences between strains were detected at many epitopes, although no correlation with clinical symptoms was made. This antigenic diversity among the multiple viral epitopes has so far yielded very little additional understanding about the biology of infection and pathogenesis.

Other less-investigated antigenic sites elicit immune responses that are not neutralizing but nevertheless contribute to serotype identity. At least one of these broad-specificity epitopes has been mapped to the amino-terminal region of the VP1 capsid protein (Roivainen et al., 1993). Although there are measurable in vitro antigenic differences among strains within a serotype, the significance of these differences during natural infection has not been determined. Several PV isolated during outbreaks have demonstrated different antigenic properties when compared with the reference vaccine strains (Huovilainen et al., 1987), but in all cases, immunity derived from vaccination has been sufficient to provide protection and control the circulation of these strains. Even in the face of massive PV immunization campaigns, no antigenic escape mutants resistant to neutralization have ever been observed, and successive genotypes of PV have been eliminated.

Virion Stability: Reactivity to Chemical and Physical Agents and Virus Storage

Many aspects of enteroviral pathology, transmission, and general epidemiology are directly related to their biophysical properties and cytolytic life cycle. The infectious virus is relatively resistant to many common laboratory disinfectants, including 70% ethanol, isopropanol, dilute Lysol, and quaternary ammonium compounds (Eggers, 1990; Narang and Codd, 1983; Poli et al., 1978; Wallbank et al., 1978). The virus is insensitive to lipid solvents, including ether and chloroform, and it is stable in many detergents at ambient temperature (Committee on Enteroviruses, 1962). Formaldehyde, gluteraldehyde, strong acid, sodium hypochlorite, and free residual chlorine inactivate EV (Saitanu and Lund, 1975; Trask et al., 1945). Concentration, pH, exogenous organic materials, and contact time affect the degree of inactivation by these compounds. Similar inactivation is achieved when virus is present on fomites, although conditions may not be exactly comparable (Abad et al., 1994, 1997). In general, most reagents that inactivate EV depend on active chemical modification of the virion, whereas most extractive solvents have no effect.

EV are relatively thermostable but less so than hepatitis A virus. Most EV are readily inactivated at 42°C, although some sulfhydral reducing agents and high-concentration magnesium cations can stabilize viruses so that they are relatively stable at 50°C (Ackermann et al., 1970; Dorval et al., 1989; Wallis and Melnnick, 1961; Wallis et al., 1965). The process of thermal inactivation appears to involve several steps, culminating in the irreversible release of RNA from the altered virion particles. The relative sensitivity to modest elevations in temperature makes it possible to use pasteurization to inactivate EV in many biologically active preparations (Stoltz et al., 1993; Strazynski et al., 2002; Uemura et al., 1994).

As with other infectious agents, UV light can be used to inactivate EV, particularly on surfaces (Benyesh et al., 1958; De Sena and Jarvis, 1981; Harper, 1961; Jensen, 1964). In addition, the process of drying on surfaces significantly reduces virus titers. The degree of virus loss by drying is related to

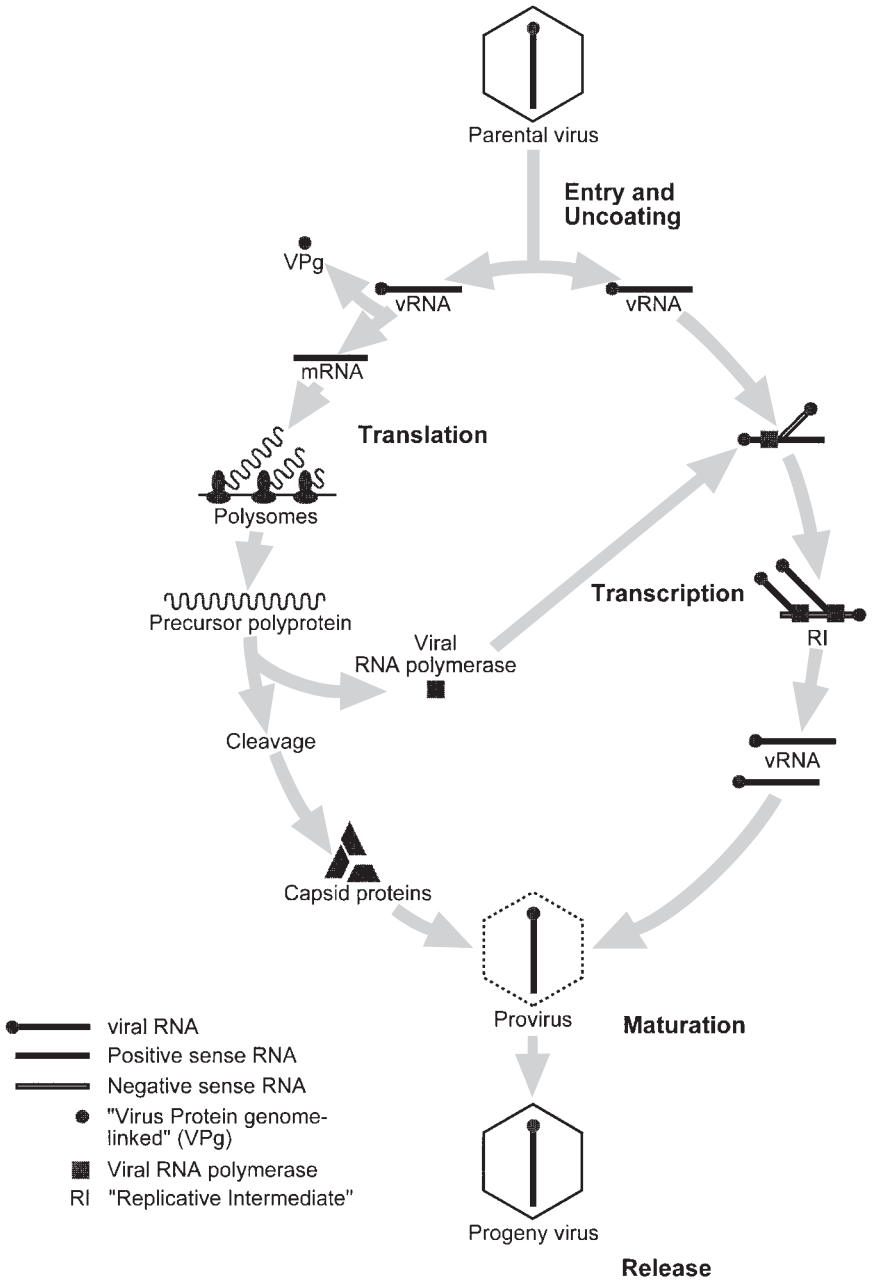

FIGURE 3 Replication cycle of PV. Parental PV enters its host cell by receptor-mediated endocytosis and releases its viral RNA from the virus capsid (uncoating) in acidic organelles (endosomes). After release of VPg from the parental viral RNA, protein synthesis of PV starts at the rough endoplasmic reticulum. The viral precursor polyprotein is autocatalytically cleaved by viral proteases, releasing in the viral RNA polymerase, and via several precursor proteins, the virus capsid proteins. At the smooth endoplasmic reticulum, the viral RNA polymerase synthesizes new viral RNA. Positive-sense RNA serves as a template for negative-sense RNA molecules, which themselves are templates for new positive-sense RNA. This positive-sense RNA is released from multistranded replicative intermediates (RI) and used for either further viral transcription and translation or encapsidation into assembling provirus particles. After the capsid protein precursor VP0 has finally been cleaved into VP2 and VP4, the maturation of progeny virus is completed. One cycle of PV reproduction takes about 6 h.

porosity of the surfaces and the presence of organic material (Abad et al., 1994).

The inactivation of infectivity may not be directly related to the destruction of the viral genome because the PCR can be used to amplify viral RNA even after inactivation of virus has occurred (Ma et al., 1994). This would suggest that reactivation of infectivity may be possible in some circumstances. In fact, some examples of recovered infectivity have been reported through increased multiplicity of infection in cell culture (Young and Sharp, 1979), but the practical significance of these observations is not clear.

Replication in Cell Culture

One of the prominent characteristics of EV is the cytolytic nature of growth in cell culture. For many years, PV was the prototype of a lytic viral infection. At the microscopic level, infection is usually manifest within 1 to 7 days by the appearance of a characteristic CPE, which features visible cell rounding and shrinking, nuclear pyknosis, refractility, and cell degeneration (Fig. 4). The earliest effects can be seen in less than 24 h if the inoculum contains many infectious particles. With fewer virions, however, visible changes are not recognizable for several days, although a sufficient number of cells are infected. In addition, some EV either do not cause CPE at all or do so only after several passages. In general, once focal CPE is detected, infection spreads rapidly throughout the cell sheet, with total destruction of the monolayer sometimes occurring in a matter of hours.

All known EV can be propagated in either cell culture or suckling mice (Melnick et al., 1979; Pallansch and Roos, 2006). Most of the serotypes can be grown in at least one human or primate continuous cell culture (Kok et al., 1998; Lee et al., 1965; Nsaibia et al., 2007; Otero et al., 2001; Patel et al., 1984; Patel et al., 1985; Pinto et al., 1994; Saijets et al., 2003; Schmidt et al., 1975). No cell line, however, can support the growth of all cultivable EV. Even after many years of experimentation, a few serotypes (e.g., CVA19) can be propagated only in suckling mice. The typical host range of human EV in cell cultures or animals is not clearly associated with a given virus species.

Infection of target cells depends on viruses binding to specific receptors on the cell surface. Collectively, the EV use at least six different receptors, including two different integrins, decay-accelerating factor (CD55), the CV-adenovirus receptor, intracellular adhesion molecule 1, and the PV receptor (CD155) (Rossmann et al., 2002). Some EV are able to use more than one receptor, and other, unidentified receptors may also exist. These host receptors may also contribute to host specificity, although studies comparing receptor homologs in resistant hosts are still in progress. A practical adaptation resulting from the identification and genetic cloning of EV receptors is the introduction of the receptor into animals and cells that do not normally permit virus infection (Huang et al., 2002; Koike et al., 1991a; Koike et al., 1991b; Mendelsohn et al., 1986; Mendelsohn et al., 1989). This approach has advanced our understanding of the pathogenesis of EV infection and found practical application in the diagnostic laboratory (Buck et al., 2002; Hovi and Stenvik, 1994; Pipkin et al., 1993; Wood and Hull, 1999).

PATHOGENESIS AND CLINICAL SYNDROMES

EV are cytopathic, and much of the associated disease presumably results from tissue-specific cell destruction. By contrast, some disease manifestations, enteroviral exanthems and myocarditis, for example, are thought to result from the host immune response to the infection (Cherry, 1993; Esfandiarei and McManus, 2008). For the most part, the actual mechanisms of virus-induced disease, however, have not been well characterized. Typically, the primary site of infection is the epithelial cells of the respiratory or gastrointestinal tract and the lymphoid follicles of the small intestine (Ouzilou et al., 2002). Replication at the primary site of infection may be followed by viremia, leading to a secondary site of tissue infection. Secondary infection of the CNS results in aseptic meningitis or, rarely, encephalitis or paralysis. Other tissue-specific infections can result in pleurodynia or myocarditis. Disseminated infection can lead to exanthems, nonspecific myalgias, or severe multiple-organ disease in neonates.

Incubation Times

All PV, CVA, CVB, and echoviruses have incubation times ranging from 2 to 35 days, with an average of 7 to 14 days (Melnick et al., 1979). The shortest incubation period, 12 to 72 h, has been reported for local infections of the eye by EV70 (Yin-Murphy, 1984).

Asymptomatic Infections

It is important to remember that the link between an EV infection and a disease syndrome should be made with caution. Inapparent infections and prolonged excretion of virus, especially in stools, are common. A definitive link cannot be made between infection and disease based solely on isolating virus from the stool of an individual patient. A link can be inferred if the virus is isolated from a site that corresponds to the clinical symptoms and if that site is normally sterile. Most associations between EV infection and disease have been made from studies of outbreaks in which a large number of persons with the same clinical signs and symptoms have evidence of infection with the same serotype. Such studies have clearly demonstrated that EV infection can cause aseptic meningitis, pericarditis, pleurodynia, myocarditis, acute hemorrhagic conjunctivitis (AHC), and encephalitis. When an individual patient has a disease syndrome shown clearly to be associated with EV infection and there is no evidence of involvement by another agent, infection implies probable causation.

Clinical Syndromes

It is neither necessary nor practical to enumerate all diseases caused by each of the EV serotypes. A limited number of viruses cause a few clinically distinct diseases (e.g., poliomyelitis, AHC, and herpangina). They are relatively easily recognized, and etiologic confirmation by laboratory tests, if required, can be directed at a few specific EV. Certain syndromes (e.g., meningitis, encephalitis, and myocarditis) have varied causes, including EV, other viruses, bacteria, and noninfectious causes, etc. In such cases, etiologic diagnosis is important for selecting appropriate treatment or avoiding inappropriate treatment. With some notable exceptions, most EV generally are capable of causing a variety of clinical diseases, and for any specific disease, it is difficult to predict the serotype from signs and symptoms alone (Table 2).

Poliomyelitis

The term poliomyelitis refers to the inflammatory damage due to infection of the anterior horn cells of the spinal cord, recognized clinically as acute-onset lower motor neuron paralysis (or paresis) of one or more muscles (Fig. 5). When its viral cause was recognized, the agent was called PV, thereby redefining poliomyelitis as spinal cord disease caused specifically

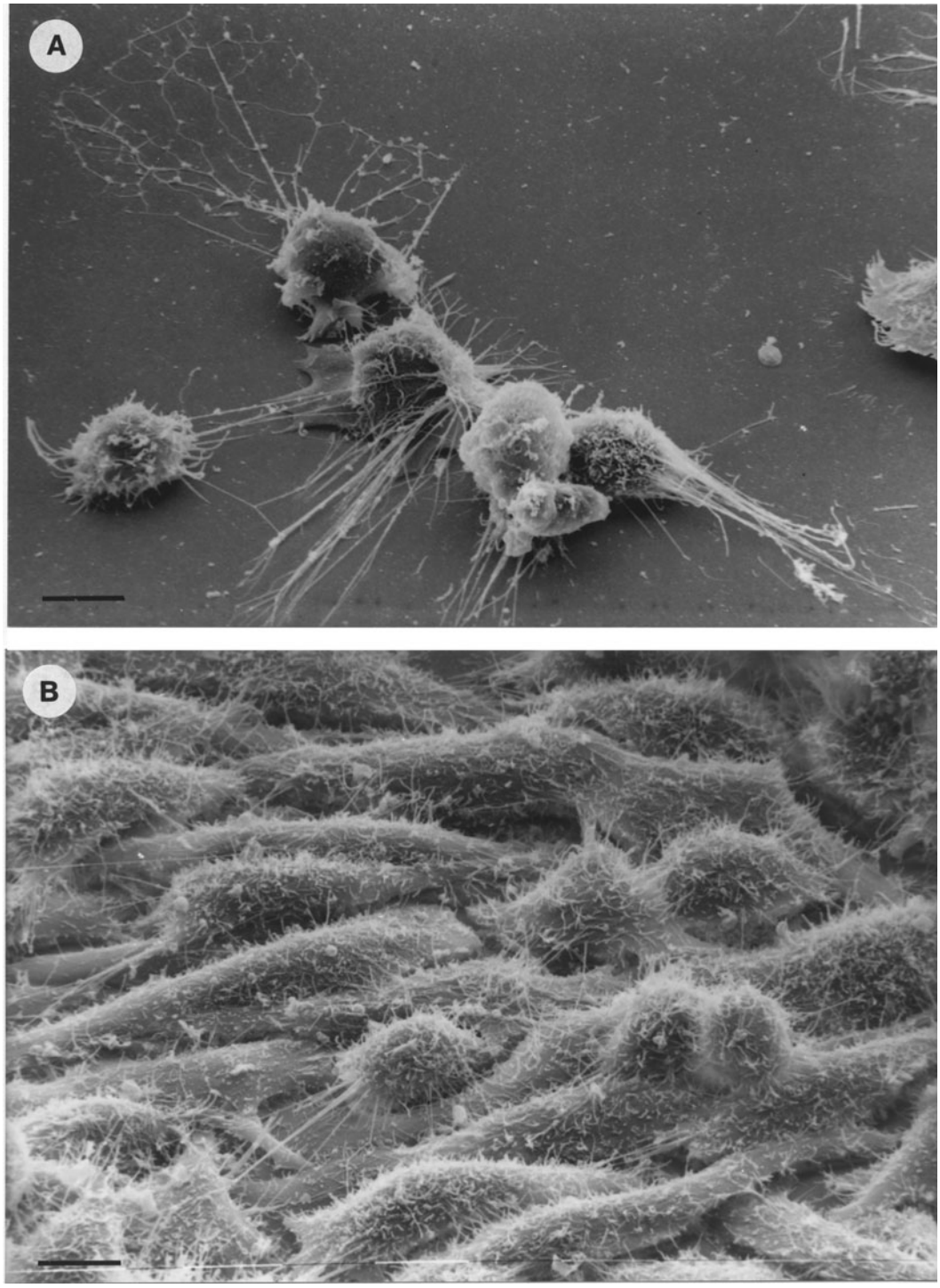

FIGURE 4 Scanning electron micrograph of HEp-2 cells infected with PV type 1. (A) Infected cells show a severe CPE, characterized by rounded-up cells that are attached to the substratum only by long filopodia. (B) Mock-infected HEp-2 cells are characterized as a monolayer of confluent cells with evenly distributed microvilli at the plasma membrane. Bars, 10 μm.

by one or another PV serotype. PV may cause other diseases, but muscle paralysis due to myelitis is the most important. Until PV infections were controlled by immunization, they were the most common cause of acute flaccid paralysis (AFP), but this is no longer the case in almost all countries.

Poliomyelitis may vary widely in severity, from paresis of one or a few muscles, or paralysis of one or more limbs, to quadriplegia and paralysis of the muscles of respiration (diaphragm, intercostal muscles). Tendon reflexes of the affected limbs are lost; in others they may be sluggish. The illness

TABLE 2 Clinical syndromes associated with EV infection

CNS
Aseptic meningitis
Encephalitis
Flaccid paralysis
Respiratory
Mild upper respiratory tract illness (common cold)
Lymphonodular pharyngitis
Bronchiolitis
Bronchitis
Pneumonia
Exanthems
HFMD
Herpangina
Cardiac
Myocarditis
Pericarditis
Other
Pleurodynia
AHC
Neonatal disseminated disease
Chronic infection of agammaglobulinemic patients

usually starts with fever and myalgia, which may last up to a week, followed by the sudden onset of paralysis that progresses to its maximum within 4 days and is typically asymmetric. Cerebral functions are not altered usually, unless hypoxia occurs. In such cases, drowsiness and occasionally mild muscle rigidity or an extensor plantar response in the unparalyzed limbs may be mistaken for upper motor neuron involvement. These signs disappear with oxygen therapy or assisted ventilation. During the acute phase of illness, the cerebrospinal fluid (CSF) shows predominantly lymphocytic pleocytosis with moderate elevation of protein. Nerve conduction studies show lower motor neuron involvement without sensory neuropathy. Most children recover from the acute illness, but some 70% continue to have some residual motor weakness, which may vary from mild impairment to complete flaccid paralysis. The permanent loss of motor neurons results in denervation atrophy of the affected muscles.

During the acute illness, cranial nerve nuclei of the medulla or higher levels may be involved, manifesting clinically as paralysis of the muscles of deglutition or central paralysis of respiration. This condition is called bulbar poliomyelitis. When spinal muscle paralysis and bulbar disease occur together, the term bulbospinal poliomyelitis is applied. Occasionally facial (seventh cranial nerve) paralysis may occur, either isolated or in combination with spinal or bulbar poliomyelitis. While the vast majority of PV infections are either asymptomatic or associated with nonspecific febrile illnesses, the case fatality rate of those who develop poliomyelitis is 2 to 5%, and in epidemics it can be as high as 10%. Death is most often due to respiratory paralysis or arrest in children with bulbar poliomyelitis.

Poliomyelitis should be considered in all cases of pure motor paralysis and is usually associated with a normal or slightly elevated value for protein, normal sugar value, and moderate mononuclear pleocytosis in CSF. Early in the illness, polymorphonuclear cells may predominate in the CSF, followed by a shift to mononuclear cells. Defects in the ventral horns of the spinal cord can be observed by magnetic resonance imaging. The magnetic resonance imaging lesion corresponds to the innervation pattern of the affected extremity. Electromyography and nerve conduction velocities generally fail to show evidence of a conduction block. The differential diagnosis includes spinal cord compression, stroke, neuropathy, and Guillain-Barré syndrome.

Delayed progression of neuromuscular symptoms (postpolio syndrome) may occur 20 years or longer after the initial paralysis due to PV (Wiechers, 1987). Postpolio syndrome is characterized by new muscle weakness associated with dysfunction of surviving motor neurons. The illness is usually associated with deterioration of those nerves involved in reinnervation during recovery from the original PV infection. Inflammation is sometimes present in association with degenerating neurons (Dalakas, 1987). It is believed that the life span of these nerves has been shortened by the process of reinnervation. This syndrome is not a form of amyotrophic lateral sclerosis. It does not appear that reactivation or replication of PV is involved, but current data are inconclusive, since no infectious virus has ever been isolated (Arya, 1997; Melchers et al., 1992).

Paralytic Myelitis Caused by Other EV

A clinical syndrome of AFP may be caused infrequently by certain EV other than PV. In children, EV71 may cause AFP either sporadically or in outbreaks (Alexander et al., 1994; Chumakov et al., 1979; Melnick, 1984; Shindarov et al., 1979). Several other EV and parechoviruses have been found to be associated with AFP on rare occasions (Figueroa et al., 1989; Gear, 1984; Grist and Bell, 1970; Hammon et al., 1958; Ito et al., 2004). The clinical picture is usually one of a mild disease, occasionally with paralysis of a single muscle, such as the deltoid, and often with complete recovery. In a few cases, particularly in adults, EV70 has been associated with meningomyelitis and AFP affecting one or more limbs (John et al., 1981; Katiyar et al., 1983; Pal et al., 1986; Saenz et al., 1984). Although most patients recover completely, some may continue to have residual paralysis, as in poliomyelitis.

Viral Meningitis

Fever, headache, and nuchal rigidity, often with Brudzinski's sign, are characteristic of enteroviral meningitis in children and adults. The CSF is usually clear, under normal or mildly to moderately increased pressure, and with mild-to-moderate pleocytosis (usual range, 100 to 1,000 cells/μl) (Graham and Murdoch, 2005; Landry, 2005; Mulford et al., 2004). Although on the first or second day of illness, CSF cells may be predominantly neutrophils, they are predominantly lymphocytes when evaluated 1 or 2 days later (Cherry, 2004). Enteroviral meningitis usually occurs sporadically, while some children are infected with the same virus without neurologic disease or even asymptomatically. Occasionally EV meningitis may occur as small outbreaks. EV are by far the most frequent cause of viral meningitis in most locations (Khetsuriani et al., 2003; Romero, 2002; Rotbart et al., 1998).

In the tropics, enteroviral meningitis occurs almost exclusively in children less than 10 years of age, similarly to poliomyelitis. In temperate regions, it may occur in children or adults, reflecting the delayed age pattern of EV infections in general. The onset is usually sudden, with fever as the first visible sign. Sometimes the fever may be biphasic: a short (1 or 2 days) febrile period accompanied by few or no other symptoms and then, after a day or two, the typical features of meningitis. In older children and adults, headache is common, along with photophobia in some. In infants and young children, febrile convulsions may occur at the onset, in which case careful examination is necessary to rule out encephalitis

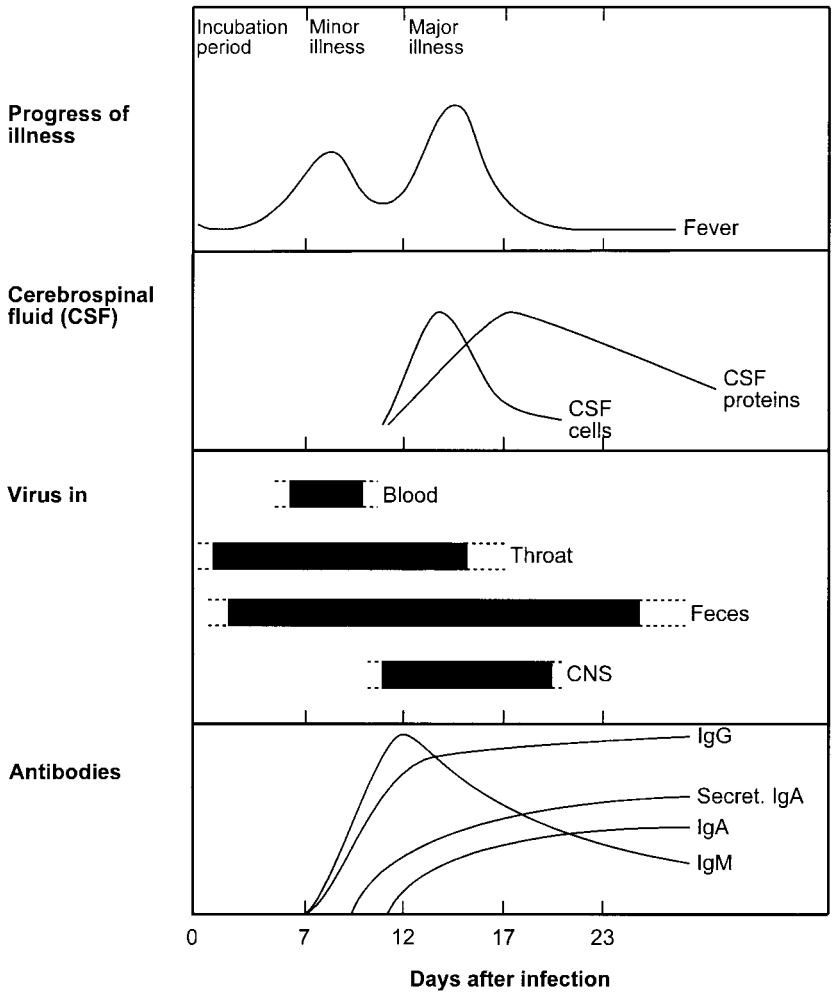

FIGURE 5 Course of PV infection.

or other CNS disease. Vomiting, anorexia, skin rash, cough, pharyngitis, diarrhea, and myalgia are also frequently present. Complete recovery is the rule. Although some data suggest that neurologic, cognitive, developmental, or language abnormalities may follow enteroviral meningitis in infancy, other studies indicate that the prognosis is more benign (Rorabaugh et al., 1993).

Many other viruses, such as mumps virus, herpes simplex virus, Epstein-Barr virus, arenavirus, and several arboviruses, also may cause viral meningitis (Baum and Koll, 2003). The clinical picture and the laboratory findings on CSF examination lead to a diagnosis of aseptic meningitis. Viral isolation in cell culture or suckling mice from CSF is usually successful only early in the course of illness. Virus isolation and detection by molecular methods are the only definitive agent-specific diagnostic results.

Encephalitis

Infection of the brain parenchyma is a relatively rare manifestation of EV or parechovirus infection (Morens and Pallansch, 1995; Pallansch and Roos, 2006; Whitley and Kimberlin, 1999). The encephalitis may be global or focal. It is probably more common in children than in adults in both tropical and temperate regions. The illness starts with fever and constitutional symptoms. After a few days, confusion, irritability, lethargy, or drowsiness develops and usually progresses rapidly to generalized convulsions and coma. In some children, focal encephalitis is characterized by focal seizures, very much as in herpes simplex virus encephalitis. Other clinical manifestations are usually related to elevated intracranial pressure and to cranial nerve or cerebellar involvement. Occasionally myelitis may also occur, with lower motor neuron paralysis of muscles (Steiner et al., 2005).

More recently, a syndrome of fatal brain stem encephalitis that is associated with EV71 infection has been described in several countries of Southeast Asia (AbuBakar et al., 1998; Chan et al., 2000; Ho et al., 1999). Although sporadic cases of infection with this virus have been described since the virus was first recognized in 1971, the deaths in Southeast Asia occurred in the context of widespread hand-foot-and-mouth disease (HFMD) outbreaks. The fatal outcome had few clinically predictive symptoms but was specifically associated with young children, most of whom were less than 2 years of age. The onset of neurologic symptoms was particularly rapid, and death often occurred within 24 h as a result of cardiopulmonary failure, presumably of neurogenic origin (Chan et al., 2000).

The CSF is usually clear, with mild, predominantly lymphocytic pleocytosis in the usual range of 5 to 200 cells/μl. The CSF protein concentration is normal or slightly elevated,

and the glucose concentration is normal or slightly decreased. As a rule, CSF culture does not yield an EV. Brain tissue is seldom obtained by biopsy for virus isolation. It is generally believed that herpes simplex virus, several arthropod-transmitted viruses, and EV are the most common causes of viral encephalitis (Lewis and Glaser, 2005; Rotbart, 1995).

Acute Myocarditis and Pericarditis and their Chronic Sequelae

Acute myocarditis, with or without pericarditis, caused by several EV can occur in infants, children, adolescents, and young adults (Magnani and Dec, 2006). The most common serotypes implicated in acute myocarditis are the CVB (Baboonian and Treasure, 1997; Diamond and Tilles, 2004; Kim et al., 2003). Myocarditis usually starts with fever and mild symptoms followed after a short interval by palpitations, chest pain, shortness of breath, and congestive cardiac failure (Dec, 2003). Occasionally, arrhythmia may be the only manifestation. Pericarditis may accompany myocarditis; often a pericardial rub is heard, but occasionally there is frank pericardial effusion. Investigation usually confirms cardiomegaly and electrocardiographic evidence of myocarditis. The case fatality rate is high enough to warrant caution in predicting prognosis (Magnani and Dec, 2006).

Treatment is supportive. Most patients recover; however, sequelae have been recognized, including chronic myocarditis, dilated cardiomyopathy, and chronic relapsing or constrictive pericarditis (Dec, 2003).

AHC

Mild conjunctival hyperemia is noted with many EV diseases. However, a severe form of conjunctivitis, usually occurring in rapidly spreading epidemics and characterized by subconjunctival hemorrhage in nearly half the subjects, is caused by two EV serotypes. This disease is different from other enteroviral illnesses, having occurred in global pandemics since its introduction around 1969, when both EV70 and "CVA24 variant" (CVA24v) emerged as causes of AHC (Kono et al., 1972; Kono, 1975; Lim, 1973; Mirkovic et al., 1973; Mirkovic et al., 1974; Yin-Murphy, 1972, 1973; Yin-Murphy and Lim, 1972). To date, AHC epidemics have occurred largely in the tropical and subtropical countries of Asia, Africa, and Latin America. Only sporadic cases or small outbreaks have occurred in temperate climates.

The illness has a sudden onset. The incubation period for these agents is shorter than for other EV (24 to 72 h), systemic illness is much less common, and conjunctival replication of virus is the rule. Spread is mainly through direct contact, via fingers or fomites. Eye pain, photophobia, excessive lacrimation, and congestion of the conjunctiva are almost always present. The characteristic subconjunctival hemorrhage in a proportion of cases in an epidemic is an important diagnostic feature. The disease is usually bilateral. Adults and school-age children are more affected than infants and preschool children, although household spread is efficient regardless of age. After a few days, symptoms abate, but the hemorrhage resolves slowly, and recovery is complete in 5 to 10 days.

During EV70 epidemics, a small proportion of subjects may develop acute paralysis of muscle groups of one or more limbs, usually within a few days after the onset of eye disease. The symptoms resemble poliomyelitis but are, in reality, a radiculomeningomyelitis (Katiyar et al., 1983). Other than this, the only common complication of AHC is secondary bacterial infection.

Pleurodynia

Pleurodynia, also known as Bornholm disease, is a distinct illness with mild or high fever of short duration and chest pain located on either side of the sternum or retrosternally, and the disease may occur sporadically or in outbreaks. The pain is usually intermittent or spasmodic and sometimes excruciating, often exacerbated by deep breathing. Intercostal muscle tenderness and a pleural rub, when present, are important signs distinguishing the illness from myocardial infarction, which is often suspected in adults. The chest radiograph and electrocardiogram are normal. Symptoms usually last for a few days to more than 2 weeks, with occasional relapses. In children, severe abdominal pain, apparently arising from the diaphragm, may occur.

This syndrome has primarily been associated with CVB (Ikeda et al., 1993; Zaoutis and Klein, 1998), particularly CVB3 and CVB5, although sporadic cases may be caused by other EV. Rarely, pleurodynia may be accompanied by another clinical manifestation of EV infection, such as aseptic meningitis or even myocarditis.

HFMD

The distinguishing feature of HFMD is the characteristic vesicular eruption on the hands and feet and in the mouth (Whiting and Smith, 1969). The oral lesions, mostly on the buccal mucosa, become shallow ulcers. CVA16 is the most frequent etiologic agent, occasionally causing large outbreaks. Other EV, especially CVA10 and EV71, may also cause HFMD outbreaks (Hosoya et al., 2006; Itagaki et al., 1983). The etiologic agent can be isolated or detected from the vesicles and from the throat and feces. Occasionally, HFMD may occur with other EV involvement such as meningitis.

Herpangina

Herpangina is characterized by a typical crop of vesicles on the soft palate, uvula, other parts of the oropharynx, or the tongue (Tunnessen, 2004). Each vesicle is about 1 to 2 mm in diameter, with a surrounding red areola. It usually occurs in children younger than 10 years old. Fever and sore throat are the common symptoms. Careful examination of the oropharynx reveals 1 to 12 discrete lesions, which usually subside without ulcerating.

Short Fever with Maculopapular Rash

Many EV may cause a short febrile illness with a maculopapular rash resembling rubella or mild measles, particularly in infants and very young children (Tunnessen, 2004). The distribution of the rash on the face, neck, and chest, and occasionally on the arms and thighs, may mimic other well-recognized exanthems. Sometimes there may be mild upper respiratory symptoms, adding to the difficulty of accurate clinical diagnosis. Outbreaks of EV exanthem also have been called Boston exanthem (Neva and Enders, 1954).

Diarrhea

Mild diarrhea is a common accompanying symptom in many enteroviral diseases, and EV may occasionally cause a short acute diarrheal illness. Although occasional outbreaks of diarrhea without other typical EV symptoms have been attributed to EV or parechovirus infections, these are uncommon.

Neonatal EV Diseases

Neonates are more vulnerable to invasive EV diseases than are older children and adults. Infection may occur in utero or, more commonly, perinatally. Many of the clinical features described previously, as well as the more sinister lesions

in the CNS or the heart, may cluster together in the infected neonate (Modlin, 1996; Verboon-Maciolek et al., 2008). Such illnesses very much resemble other severe systemic infectious diseases, such as bacterial septicemia. Thus, the infant may present with lethargy, feeding difficulty, vomiting, tachycardia, dyspnea, cyanosis, jaundice, and diarrhea, with or without fever. Clinical evidence for aseptic meningitis, encephalitis, myocarditis, hepatitis, or pneumonia may be present in any combination. The case fatality rate is high. Sometimes death may occur rapidly.

Respiratory Disease

Like the related human rhinoviruses, EV are often associated with mild respiratory illness, most frequently restricted to the upper respiratory tract (common cold) (Portes et al., 1998). However, EV and parechoviruses may also cause more serious, lower respiratory tract illness, including bronchitis, bronchiolitis, and pneumonia (Chung et al., 2007; Jacques et al., 2008). In particular, EV68 appears to be almost exclusively restricted to the respiratory tract and associated with respiratory disease (Oberste et al., 2004b).

IMMUNE RESPONSE

Innate Immune System

The efficacy of the immune response is key to the outcome of enteroviral infections. The innate immune system is especially important because it is the earliest response and, in addition, it regulates the adaptive immune response. A recent study suggests that the innate immune system is an important determinant of tissue tropism and pathogenicity of PV (Ida-Hosonuma et al., 2005). Interferons (IFN) stimulate many genes that induce an antiviral state. In the case of CVB4-infected pancreatic islet cells, IFN-α induced 2′,5′-oligoadenylate sythetases and ribonuclease L (RNase L), which protected cells against the infection, whereas IFN-γ induced double-stranded RNA-dependent RNA-activated protein kinase (Flodstrom-Tullberg et al., 2005). One of the factors that activates IFN-β (and leads to the expression of a number of proinflammatory cytokines) and is also induced by protein kinase is nuclear factor kappa B (NF-κB). In vitro studies have shown that NF-κB is activated early after PV infection. Later, the effect of NF-κB is counteracted through proteolytic cleavage of the p65-RelA subunit of NF-κB by means of the PV 3C protease (Neznanov et al., 2005); in addition, the PV 2A protease-mediated inhibition of host cell translation interferes with the downstream effects of NF-κB. The role of innate lymphocytes and natural killer cells has been explored to a relatively small extent. These cells may play a role not only in protection but also as immune mediators of the disease process (Dotta et al., 2007; Gauntt et al., 1989; Godeny and Gauntt, 1986, 1987; Huber, 2008; Mandelboim et al., 1997).

B-Cell Response

The humoral immune response in enteroviral infections is especially important for protection and life-long immunity. For example, a high level of PV neutralizing antibody following natural infection or vaccination protects against disease, although infection of the gut can occur—that is, there is no sterilizing immunity. The importance of the humoral immune response in enteroviral infections is demonstrated by the occurrence of persistent EV infections in agammaglobulinemic patients and by the increased susceptibility to enteroviral infection, as well as the increased severity of enteroviral disease, among neonates and young infants.

T-Cell Response

There are several mechanisms by which T cells can lead to protection and immunity from EV infection. $CD4^+$ T cells may be important in providing help for the B-cell humoral immune response which, as noted, is critical for EV clearance. Cytolytic T cells may clear the virus directly by causing lysis of virus-infected cells or indirectly by means of cytokine release. Gamma-delta T cells and natural killer cells, which are part of the innate immune response, may regulate the adaptive immune T-cell response and thereby play an important role in virus clearance and potentially in immune-mediated pathology.

Immunopathology

Although the B- and T-cell responses in EV infections both are important in virus clearance, there are indications that the immune response may also lead to disease in certain situations (Whitton, 2002; Whitton et al., 2005). There are several reasons for considering a role for immunopathology in some EV-induced diseases: (i) some EV-induced diseases have inflammatory pathology; (ii) the experimental animal models for these EV-induced diseases demonstrate the importance of genetic susceptibility in their causation and the efficacy of treatment with immunosuppressive drugs; and (iii) certain EV-induced diseases are considered to have an autoimmune component (Rose, 2008). Immune-mediated disease processes have been most vigorously proposed for EV-induced chronic dilated cardiomyopathy lesions, chronic myositis, and type I diabetes mellitus (Drescher and Tracy, 2008; Knowlton, 2008; Ramsingh, 2008).

Molecular mimicry and bystander damage have been proposed as potential mechanisms of virus-induced autoimmune disease (Fujinami et al., 2006). In the former, an immune response directed against the virus cross-reacts with a host cellular protein and causes disease. In the case of bystander damage, a virus infection up-regulates nonspecific immune responses, leading to nonspecific, generalized pathology in the infected tissue. Recent data regarding CV-induced insulin-dependent diabetes mellitus and CV-induced myocarditis suggest the importance of bystander damage rather than molecular mimicry (Horwitz et al., 1998). The activation of autoreactive resting T cells could be induced by cytokines or other factors, such as the release of self-antigen (Horwitz et al., 2000). The presence of proinflammatory cytokines would then activate Th1 cells or macrophages that foster immunopathology. Once damage has occurred, new cellular epitopes that are not normally exposed may trigger an immune response that leads to tissue damage, with the further unmasking of additional reactive epitopes, a process called epitope spreading (Fujinami et al., 2006).

EPIDEMIOLOGY

Mode of Transmission

EV can be isolated from both the lower and upper alimentary tract and can be transmitted by both fecal-oral and respiratory routes. Fecal-oral transmission may predominate in areas with poor sanitary conditions, whereas respiratory transmission may be more important in more developed areas (Horstmann, 1967). The relative importance of the different modes of transmission probably varies with the particular EV type and the environmental setting. It is believed that almost all

EV, except possibly EV68 and EV70, can be transmitted by the fecal-oral route; however, it is not known whether most can also be transmitted by the respiratory route. EV70 and CVA24v, the agents that cause AHC, are seldom isolated from the respiratory tract or stool specimens, and are probably primarily spread by direct or indirect contact with eye secretions (Kono, 1975). EV that cause a vesicular exanthema presumably can be spread by direct or indirect contact with vesicular fluid, which contains infectious virus. It is likely that EV are transmitted in the same manner as are other viruses causing the common cold—that is, by hand contact with secretions (e.g., on the hand of another person) and autoinoculation to the mouth, nose, or eyes. Direct bloodstream inoculation, usually by laboratory accidents (e.g., needle sticks) can result in EV infection. EV are efficiently amplified and transmitted among humans without intermediaries such as arthropods or other animals. Although several species of nonhuman primates may become experimentally or even naturally infected with some human EV, there is no evidence that they play any significant role in virus circulation or constitute an effective animal reservoir. EV have been detected in pooled donor blood (Welch et al., 2003; Welch et al., 2001), but neither blood transfusion nor mosquito or other insect bite appears to be a significant route of transmission.

Transmission within households has been well studied for both PV and nonpolio EV. Small children generally introduce EV into the family, although young adults make up the majority of index cases in some outbreaks of AHC (Sawyer et al., 1989). Intrafamily transmission can be rapid and relatively complete, depending on duration of virus excretion, household size, number of siblings, socioeconomic status, immune status of household members, and other risk factors (Hall et al., 1970). Transmission generally has been found to be greatest in large families of lower socioeconomic status, with a greater number of children 5 to 9 years of age, and with no evidence of immunity to the virus type. Not surprisingly, infections in different family members can result in different clinical manifestations.

Observations of household transmission of various EV have documented that many infected contacts do not become ill and that the extent of secondary transmission varies with different EV. Household secondary attack rates in susceptible members may be greatest for the agents of AHC and for the PV and of lesser magnitude for the CV and echoviruses. In some studies, secondary attack rates may be ≥90%, although they are typically lower. Data from the New York Virus Watch study indicated that EV infections were more frequent among children 2 to 9 years of age and that secondary CV infections were more frequent in mothers (78%) than in fathers (47%) (Kogon et al., 1969). In the same study, CV spread to 76% of exposed susceptible persons versus 25% of exposed persons who had detectable antibody to the infecting type; echoviruses infected 43% of those who were susceptible and only one person who had antibody. The greater spread of PV and CV may derive from longer periods of virus excretion.

Transmission occurs within the neighborhood and community, particularly where people congregate. EV can be rapidly transmitted within institutions when circumstances permit (e.g., crowding, poor hygiene, or contaminated water), similar to many other viruses. School teams or activity groups and institutionalized ambulatory retarded children or adults may be at special risk (Alexander et al., 1993). Despite crowding, EV transmission is not usually accelerated to a noticeable degree in institutions where good sanitation is found.

Nosocomial transmission of various EV and parechoviruses also has been well documented, typically in newborn nurseries. Hospital staff may have been involved in mediating transmission in some of these outbreaks. EV70 and CVA24v are highly transmissible and can cause outbreaks in ophthalmology clinics when instruments are inadequately cleaned between patients. An apparent outbreak of CVA1, which included some fatal cases, has been reported in bone marrow transplant recipients (Townsend et al., 1982).

EV have been found in surface and ground waters throughout the world. In the tropics, virus survival is more prolonged in groundwater because it is cooler than surface water. EV do not generally survive in the environment beyond a few weeks, although EV can survive for months under favorable environmental conditions; these favorable conditions include neutral pH, moisture, and low temperatures, especially in the presence of organic matter, which protects against inactivation. Although little evidence suggests that EV found in the environment are of public health importance, concern has been expressed about the possible dangers of contaminated water sources. Recreational swimming water has been investigated in several studies, and EV have been isolated from swimming and wading pools in the absence of fecal coliforms and in the presence of recommended levels of free residual chlorine (Keswick et al., 1981). Reports have suggested that swallowing of contaminated pool or lake water, theoretically, may account for transmission, but no proof exists that this type of transmission is significant in recreational settings. In industrialized countries, EV transmission from potable water is apparently uncommon, but it is a constant source of concern for public health investigators because the usual conditions under which municipal drinking water is chlorinated may be insufficient to completely inactivate EV.

EV have been isolated from raw or partly cooked mollusks and crustaceans and their overlying waters (Goyal et al., 1979). Shellfish rapidly concentrate many viruses, including EV. These viruses can survive in oysters for 3 weeks at temperatures of 1 to 21°C, but to date, no outbreak of EV disease has been attributed to the consumption of contaminated shellfish. Other food-borne transmission has been documented but is thought to be uncommon. A 1976 outbreak of aseptic meningitis attributed to E4 was apparently caused by consumption of contaminated coleslaw at a large picnic (Centers for Disease Control and Prevention, unpublished data).

EV are more prevalent in sewage from areas with low socioeconomic conditions or with large proportions of young children. In addition, sewage workers have been shown to have a higher prevalence of serum antibodies to EV, consistent with an occupational risk (Clark et al., 1977). Soil and crops also provide conditions favorable to EV. EV survive well in sludge and remain on the surface of sludge-treated soil and even on crops. Air samples from aerosolized spray irrigants using contaminated effluents also have been found to contain EV (Moore et al., 1979). Survival of EV on vegetable food crops exposed to contaminated water or fertilizer has not been proven to be associated with virus transmission.

In tropical regions, especially where sanitation is poor, the efficiency of transmission is high. Consequently, not only is the overall prevalence of EV infections higher but also the average age of infection is younger. During infancy and preschool age, children get frequent infections with many EV (Feldman et al., 1970; John et al., 1978). It is not uncommon in these areas to detect two or three simultaneous infections of different EV serotypes, often causing no disease. Since children likely become infected with nearly all EV in the tropics,

most adults are immune and nearly all babies are born with maternal antibodies to most EV (Mukundan and John, 1983). Normally, neonates not protected by maternal antibodies are at risk for serious illness when infected by EV. Because of the protective maternal antibodies, neonatal EV disease is extremely rare in the tropics, despite the high prevalence of EV infections in older children. In contrast, in temperate regions where the overall prevalence of EV infections and maternal antibodies are much lower, neonatal EV diseases are relatively more common (Morens, 1978).

It is important to note that shedding may be intermittent and is affected by the immune status of the individual. Past natural infection with the same EV serotype (or immunization, in the case of PV) serves to significantly reduce the extent and duration of virus shedding (Ramsay et al., 1994). Immunity will protect against disease but does not form an absolute block that will prevent future infection. Therefore, immune individuals can also contribute to virus transmission, while not being at risk for significant disease.

Geography, Season, Socioeconomic Factors, Sex, Age, and Risk Groups

EV excretion does not necessarily imply association with disease because most such excretion is asymptomatic. EV activity in populations can be either sporadic or epidemic, and certain EV types are associated with both sporadic and epidemic disease occurrences (Khetsuriani et al., 2006). The reported incidence or prevalence of a given EV disease may artifactually be increased in an outbreak situation when a sudden focus of attention improves diagnosis and reporting of cases, but this may also increase reporting of noncases. In addition, there may be a tendency for other strains to be excluded when a particular strain is predominant in a community; however, large communities with summer enteroviral disease typically support cocirculation of several different types simultaneously and in no particular pattern (Parks et al., 1967).

Incidence data for diseases caused by particular EV types can be derived from prospective longitudinal surveillance of a defined population or from a sample of the population in which the occurrence of disease or infection can be reliably determined (Gelfand et al., 1958). Less-useful information is based on passive case finding. Because such data indicate neither how many ill persons were not reported nor how many ill persons had negative laboratory tests, the information is mostly of qualitative value; however, it may be useful in indicating trends (Moore, 1982; Morens et al., 1979). In the United States, EV surveillance data are collected and analyzed by the Centers for Disease Control and Prevention (CDC). The data have been reported irregularly since the beginning of the program in 1961, but a 36-year report has recently been published (Khetsuriani et al., 2006). In the United States, the only notifiable enteroviral diseases are poliomyelitis and encephalitis. These are reportable by diagnosis only (e.g., encephalitis) and not etiology (e.g., echovirus encephalitis).

An important concept in understanding the epidemiology of the EV is variation: by serotype, by time, by geographic location, and by disease. This concept is illustrated in surveillance studies of nonpolio EV infections. For example, Fig. 6 summarizes the data for the years from 1970 to 2005 for CVB3, E9, and E30 isolates in the United States collected and analyzed by the CDC. These data illustrate endemic and epidemic patterns of EV prevalence. The epidemic pattern, as typified by E30, is characterized by peaks in numbers of isolations followed by periods with few isolations. These peaks may be sharp (1- or 2-year) or broad (multiyear) periods of increased virus isolations. For example, during the period from 1970 to 2006, several major E30 epidemics occurred in the United States: from 1981 to 1982, 1990 to 1994, and 1997 to 1998 (Helfand et al., 1994; Leonardi et al., 1993; Mohle-Boetani et al., 1999). By contrast, endemic viruses (e.g., CVB3) are isolated nearly every year and in similar numbers each year. Even with viruses endemic to an area, larger outbreaks do occasionally occur, as with CVB3 in 1980. Similar endemic and epidemic patterns are seen for the other echoviruses and CVA (Khetsuriani et al., 2006).

Variation by location is also a major characteristic of EV. Outbreaks can be restricted to small groups (e.g., schools and day care centers) or to select communities, or they may become widespread at the regional, national, or even international level. Outbreaks in small groups can sometimes be linked epidemiologically to a breakdown in hygiene practices. Even during national outbreaks of a specific serotype, the location of virus activity may not be uniform. During the period from 1990 to 2005, E30 was the most commonly isolated EV in the United States; however, not all parts of the country had E30 isolates during the entire period. Some areas, such as the New England states, had extensive circulation in only 1 year, whereas other areas, such as the entire western United States, had extensive virus circulation at least 3 of the 4 years. It is important to note, therefore, that aggregate national data can obscure significant regional and local variation in viral prevalence.

In temperate climates, EV are characteristically found during the summer and early autumn, although outbreaks can continue into the winter. In fact, naturally occurring EV have a distinct seasonal pattern of circulation that varies by geographic area; in contrast, live, attenuated PV vaccine strains are isolated year round in OPV-using countries, reflecting the routine administration of polio vaccine to children. In tropical and semitropical areas, circulation tends to be year-round or associated with the rainy season. In the United States, 36 years of surveillance indicated that 82% of EV isolations were made during the five summer or fall months of June to October (Khetsuriani et al., 2006). In a 6-year study of viral CNS disease, 85% of enteroviral disease, compared with 12 to 26% of diseases caused by other viral agents, occurred between June and November (Moore, 1982).

Many studies have examined the prevalence of antibodies to the EV in specific populations, with several important conclusions (reviewed in Melnick, 1996). First, the number of persons who have neutralizing antibody to any given EV is large, indicating a high incidence of past infection. A high incidence of recent infection is also suggested by surveys of immunoglobulin M (IgM) antibodies to EV, which typically show 4 to 6% positivity. Second, infections with one serotype of EV can boost antibody titers to other EV serotypes as measured by either IgM or neutralization. The pattern of the heterotypic response varies by serotype and among individuals. The nature of this heterotypic response has been explored through the identification of specific epitopes using monoclonal antibodies and peptide antisera (Samuelson et al., 1994). Third, the pattern of antibody prevalence by serotype varies by geographic location, time, and age. Thus, prevalence data from different years and locations are not directly comparable. These three points must be considered when interpreting the findings of serologic studies of associations between EV infection and disease.

A specific risk group occurs as a result of widespread but incomplete PV immunization when PV-susceptible enclaves arise. These usually consist of unvaccinated religious groups in

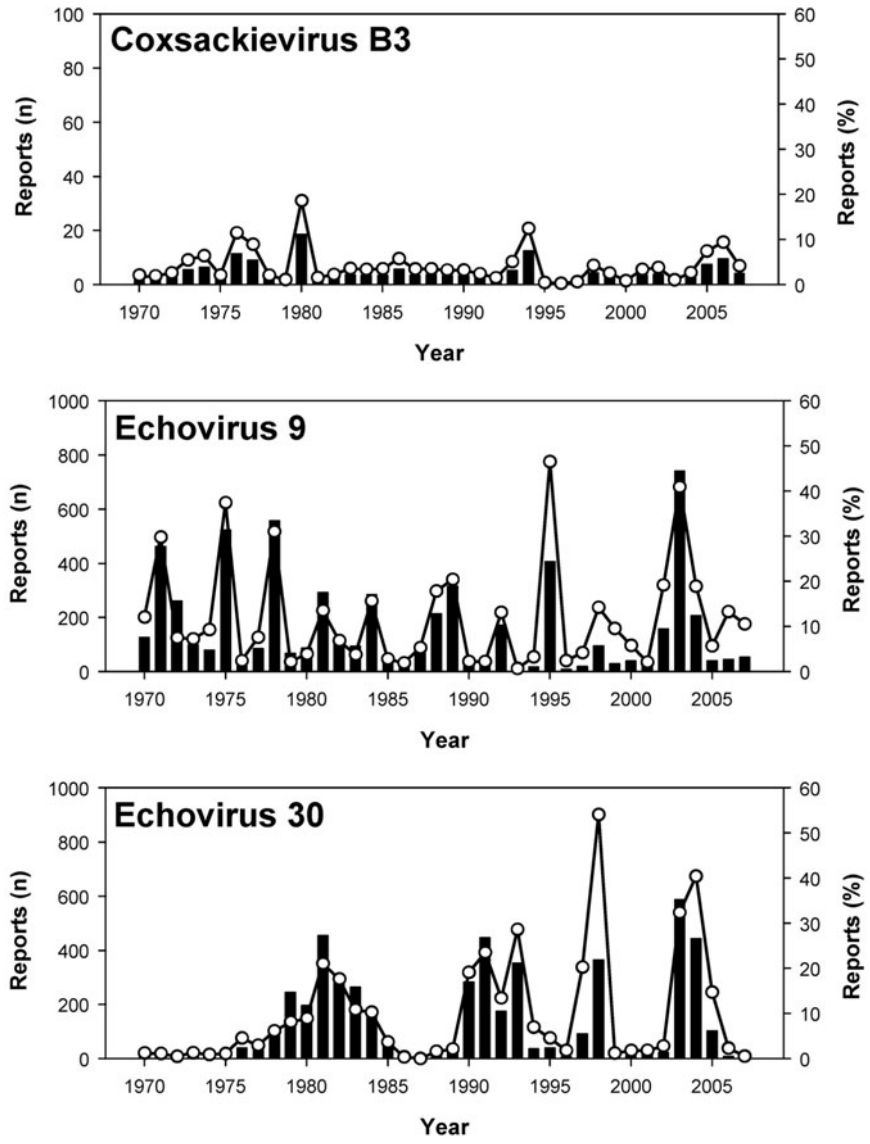

FIGURE 6 Temporal prevalence of E9, E30, and CVB3 in the United States from 1970 to 2005. The graph shows the number of isolates (bar graph) and the fraction of all nonpolio EV (line graph) that each of the serotypes represents in each year.

countries with an otherwise high prevalence of PV immunity. Despite the barrier of millions of immune persons, PV outbreaks have occurred in some of these enclaves (Conyn-van Spaendonck et al., 1996). This suggests that herd immunity may be of only limited value in protecting groups of susceptible persons who have regular contact with outside populations, and it raises questions about the risks that such groups may pose to the community at large (Anonymous, 1993).

Molecular Epidemiology

Study of the molecular variation of viral proteins or nucleic acid may contribute significant epidemiologic information on viral diseases. Molecular epidemiologic studies have helped in our understanding of EV by providing the opportunity for unequivocal strain identification, providing insights into EV classification and taxonomy, clarifying the origins of outbreaks, and allowing identification of strains transmitted between outbreaks. For the EV, and in particular PV, the primary method used to generate epidemiologic information is direct analysis of genomic variation using nucleic acid sequencing (Kew and Nathanson, 1995; Kew et al., 1995b). Nucleic acid sequencing technology has been most comprehensively applied to studies of PV, where the information has proven valuable for supporting the global PV eradication program. The introduction of the technique of genomic nucleic acid sequencing and its application to the study of wild PV isolates from different parts of the world has significantly extended the epidemiologic power of molecular studies. By analyzing the random mutations that occur in the genome of different PV, closely related viruses were easily differentiated and, in addition, more distantly related viruses were clustered into distinct geographic groupings based on endemic circulation (Rico-Hesse et al., 1987). This approach allowed epidemiologic links to be extended beyond those identified with other techniques. Building on a nucleic acid sequence database of PV strains worldwide, it has been

possible to develop rapid approaches to tracking wild PV strains (Centers for Disease Control and Prevention, 2007a).

Studies on the molecular epidemiology of nonpolio EV have focused on the evolutionary inference derived from the comparison of virus isolates within a serotype over time as well as the comparison of isolates from different serotypes and even between different genera within the *Picornaviridae*. Molecular epidemiologic studies using sequencing have been reported for CVA9 (Santti et al., 2000), CVA16 (Hosoya et al., 2007; Perera et al., 2007), CVA24 (Brandful et al., 1991; Dussart et al., 2005; Ishiko et al., 1992a; Ishiko et al., 1992b; Leveque et al., 2006; Lin et al., 1991; Lin et al., 1993; Lin et al., 2001; Park et al., 2006; Tavares et al., 2006; Triki et al., 2007; Yeo et al., 2007), CVB1 (Zoll et al., 1994), CVB4 (Hughes et al., 1993; Mulders et al., 2000), CVB5 (Kopecka et al., 1995; Papa et al., 2006; Rezig et al., 2004), E4 (Handscher et al., 1999), E11 (Chevaliez et al., 2004; Kapoor et al., 2004; Lukashev et al., 2002; Lukashev et al., 2003; Oberste et al., 2003; Szendroi et al., 2000), E13 (Avellon et al., 2003; Diedrich and Schreier, 2001; Kaida et al., 2004; Mullins et al., 2004), E19 (Lukashev et al., 2002; Zhu et al., 2007), E30 (Bailly et al., 2000; Bailly et al., 2002; Gjoen et al., 1996; Lukashev et al., 2004; Lukashev et al., 2008; Mirand et al., 2007; Oberste et al., 1999a; Paananen et al., 2007; Palacios et al., 2002; Savolainen et al., 2001; Vestergaard et al., 2004; Wang et al., 2002a; Yoshida et al., 1999; Zhao et al., 2005), E33 (Huang et al., 2003), EV68 (Oberste et al., 2004b), EV70 (Shulman et al., 1997; Takeda et al., 1994), and EV71 (AbuBakar et al., 1999; Brown et al., 1999; Cardosa et al., 2003; Hosoya et al., 2006; Li et al., 2005; McMinn et al., 2001; Mizuta et al., 2005; Shih et al., 2000; Shimizu et al., 1999; Shimizu et al., 2004b; Singh et al., 2000; Wang et al., 2002b; Witso et al., 2007; Yan et al., 2000). One of the studies of CVB5 isolates examined the pattern of genetic changes over three separate outbreaks in the United States (Kopecka et al., 1995). The nucleotide sequence from multiple isolates from the epidemics showed that each of the epidemics was caused by a single genotype. The genotype of CVB5 observed in the 1967 epidemic showed more similarity to the virus observed in the 1983 epidemic than to viruses isolated during the intervening years, suggesting discontinuous transmission of epidemic CVB5 in the United States during this time. In an analogous manner, E30 genotypes have demonstrated an overlapping succession among the isolates characterized in the United States (Oberste et al., 1999a).

More than one genotype may be found in certain periods, and the displaced genotype can be found in other parts of the world after isolations have ceased in the United States for many years. In studies of EV71 isolates, at least three distinct genotypes, and possibly a fourth, have been characterized (Brown et al., 1999; Hosoya et al., 2006; Lin et al., 2006). Unlike the situation with E30 and more similar to the CVB5 example, the transition from one genotype to another occurred during a single year, 1987, and the older genotype has not been isolated in the United States since then, despite isolation in other parts of the world.

LABORATORY DIAGNOSIS

A general diagnostic caveat is shared among EV and other ubiquitous pathogens. Since EV infections are quite common, especially in childhood, and since most infections are noninvasive and prolonged, the detected EV infection need not be the cause of the illness under investigation. Rather, it is an issue of probabilities. If the clinical syndrome is already known to be associated with the detected agent, then infection is taken as reasonable evidence of causation. If the presence of the agent is found in diseased tissue or a relevant body fluid (such as CSF), then that constitutes concrete evidence of invasion and, hence, causation (Table 3).

Virus Isolation and Identification

Many of the detailed procedures for the laboratory diagnosis of EV and parechovirus infections using virus isolation have been described previously (Melnick et al., 1979). The traditional techniques for detecting and characterizing EV rely on the time-consuming and labor-intensive procedures of viral isolation in cell culture and neutralization by reference antisera. Isolation of EV from specimens using appropriate cultured cell lines is often possible within 2 or 3 days and remains a very sensitive method for detecting these viruses. The best specimens for isolation of virus are, in order of preference, stool specimens or rectal swabs, throat swabs or washings, and CSF. Throat swabs or washings and CSF are most likely to yield virus isolates if they are obtained early in the acute phase of the illness. For cases of AHC, the best specimens are conjunctival swabs (Melnick et al., 1979), although occasionally virus can be isolated from tears (Shulman et al., 1997). Since the major pandemic in 1981, however, isolation of EV70 from patients with AHC has been very difficult, and molecular methods provide the only sensitive method to detect this agent (Shulman et al., 1997).

The procedure for virus isolation involves inoculation of appropriate specimens onto susceptible cultured cells. No single cell line, however, exists that is capable of growing all human EV. It is common practice to use several types of human and primate cells to increase the spectrum of viruses that can be detected (Melnick et al., 1979; She et al., 2006). Even with a variety of cells, however, several CVA serotypes fail to propagate in culture (Schmidt et al., 1975). The CV, including those that do not grow in cell culture, can be

TABLE 3 Preferred methods for picornavirus detection and characterization

Detection	Sensitivity	Specificity	Ease of use	Speed
Cell culture	++	+	++	++
Suckling mouse inoculation	++	+	+	+
RT-PCR	+++	++	+++	+++
Identification/characterization				
Neutralization				
Individual antisera	++	+++	+	+
Pooled antisera	++	++	++	+
Indirect immunofluorescence	++	++	+++	++
PCR + sequencing	+++	+++	+	++

isolated and propagated by intracerebral inoculation of suckling mice (Schmidt et al., 1975). The nature of the CPE in various cell cultures is so characteristic that a cautious presumptive diagnosis of EV infection can often be made at initial detection of cytopathic changes. Some components of a sample inoculated onto cell cultures (from fecal samples, for example) can produce cell toxicity in the first 24 h, which can be confused with viral CPE. To distinguish toxicity from virus CPE, an additional passage can be performed so that toxic components of a specimen can be diluted; the passage will allow cells to maintain viability or, alternatively, for the virus to amplify and produce CPE.

As a consequence of current PV eradication activities and the importance of PV as a public health problem, specific diagnostic procedures have been developed to detect this virus (World Health Organization, 2001). In general, PV grows well on a variety of primate and human cell culture lines, but it cannot be distinguished from other EV solely on the basis of CPE. PV are unique in their use of CD155, which is distinct from receptors used by all other EV to infect cells. This receptor has been transfected and expressed in a murine cell line that normally cannot be infected by most EV but is permissive to viral replication when the viral genome is present within the cell. One of these transfected murine cells, L20B, can grow PV, and has been exploited selectively to isolate PV, even in the presence of other EV (Pipkin et al., 1993; Wood and Hull, 1999). When a specimen is inoculated onto these cells and a characteristic EV CPE is seen, the virus can be presumptively identified as a PV. A few strains of certain nonpolio EV serotypes are able to grow on the parent murine L cells, however, and therefore, growth on L20B cells is not a definitive identification of PV and confirmatory testing is required.

In routine diagnostic testing, all EV growth in cell culture is detected by its CPE, and the isolate is typically confirmed as a specific EV by neutralization with type-specific antisera. The most widely used reagent antisera were prepared in horses and can be obtained from the World Health Organization (WHO) (Hampil and Melnick, 1968; Melnick and Hampil, 1965, 1970, 1973); however, supplies are now becoming limited. Type-specific monoclonal antibodies may also be used for EV typing. Typically, they are used in indirect immunofluorescence assays to identify viruses isolated in cultured cells (Lin et al., 2008; Rigonan et al., 1998). Commercially available monoclonal antibodies can be used to detect relatively common serotypes, including PV1 to 3, CVA9, CVA24, CVB1 to 6, E4, E6, E9, E11, E30, EV70, and EV71. Additional monoclonal antibodies have recently been developed for CVA2, CVA4 to 5, and CVA10 (Lin et al., 2008). Indirect immunofluorescence is faster and easier to perform than neutralization and the reagents can be produced in large quantity as needed, but the method still suffers from the same limitations as other antigenic typing methods, namely the requirement for a virus isolate in culture prior to typing and the need for a large number of reagents to identify all serotypes. Despite these limitations, the method has been adopted as the standard EV typing method in a large number of clinical and reference laboratories.

Because of the large number of known EV serotypes, it would be impractical to attempt neutralization using all possible reference antisera individually. Type-specific antisera, therefore, are combined in intersecting pools such that antibody to any one type is present in only a limited number of pools. In the Lim and Benyesh-Melnick pool scheme (Lim and Benyesh-Melnick, 1960), antibodies to 42 different EV are sorted into eight pools (labeled A through H) (Fig. 7). Even when using these procedures, untypeable EV are encountered. They may represent virus serotypes not included in the pools, mixed infections, aggregates of viruses, extreme antigenic drift, or undiscovered types. In theory, it should be possible to identify or characterize all of these, but such laborious and time-consuming processes are rarely justified. For clinical management of routine cases, it is seldom critical to identify the specific nonpolio EV type. A high index of suspicion for an EV infection can be developed by reflecting on the clinical picture, the virus isolate's CPE and cell culture systems utilized, and knowledge of basic EV epidemiology.

Molecular Detection and Characterization

The application of molecular biology techniques to clinical virology has begun to significantly change approaches to EV diagnostics. Because of distinct advantages in speed, many of these procedures have already supplanted traditional methods of detection and characterization. Specifically, molecular procedures are now the methods of choice for EV detection in CSF and are widely used for a patient with a clinical presentation of meningitis.

As with virus isolation and serotyping, the molecular methods attempt to detect the presence of EV in a specimen and, in some procedures, to further characterize the detected virus. The techniques can be grouped on the basis of their infrastructure and technical requirements and the types of specimens to which they are applied. Additionally, the tests can be grouped on the basis of the type of answer the test provides and the predictive value of a positive and a negative finding. The first broad group of tests is based on PCR, which is used primarily to detect EV genomes in cell cultures, clinical specimens, and biopsy or autopsy tissues. The second and newest procedure utilizes genomic sequencing for the characterization of EV at the highest levels of specificity.

By far the most common use of PCR for EV diagnosis is the direct detection of virus in clinical specimens (Romero, 1999; Sawyer, 2001). Numerous variations on the details of the procedures are found, but all methods that can generically detect EV have several common features. The most important property of these tests is that the primers are targeted to amplify the 5′ NTR of the virus genome. Many different primers targeted to this region have been published, and they target different sequences and provide different product sizes (Oberste and Pallansch, 2005; Rotbart and Romero, 1995). Many of these primers, however, have not been completely evaluated on a large number of clinical isolates to confirm reactivity with all EV serotypes and strains within serotypes, and therefore, they have not been validated sufficiently for diagnostic use (Oberste and Pallansch, 2005). The major advantage of the pan-EV PCR is that rapid detection of an EV is possible, even with very small amounts of clinical specimens such as CSF. It is also possible to detect EV that do not readily grow in cell culture. As with all PCR, the sensitivity of amplification of RNA from biologic specimens is extremely variable, depending on the nature of the specimen. Although the PCR procedure can be shown to give a positive result even from only one or a few copies of viral RNA, it is not unusual for the sensitivity to be many orders of magnitude lower in certain specimens (e.g., stool). The introduction of "real-time" PCR methods has largely solved this issue, as the fluorescence detection systems generally increase the sensitivity significantly.

A goal in virus identification is knowledge of the sequence of the viral genome. Encoded within this sequence are determinants for all the biologic properties that are attributable to a given virus. Therefore, the nucleic acid sequence

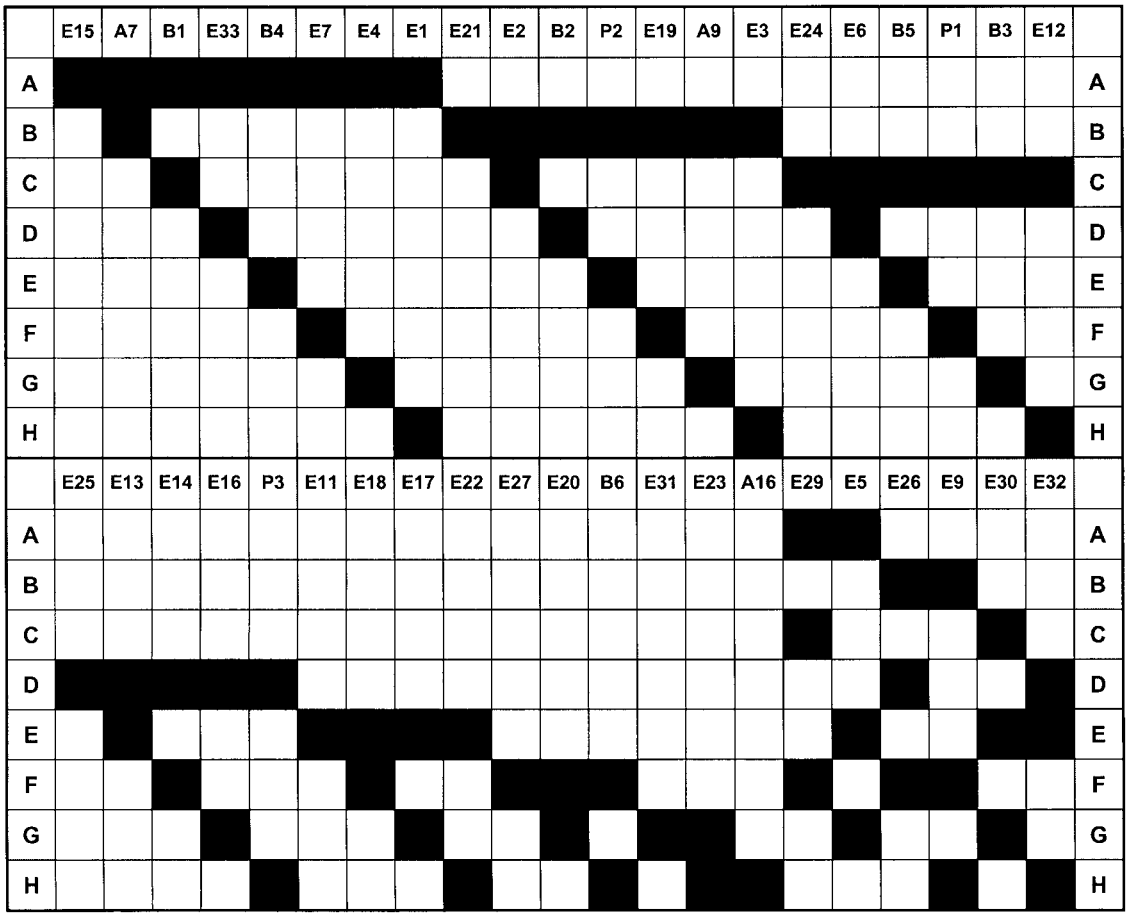

FIGURE 7 Lim and Benyesh-Melnick antiserum pools for typing of EV. Filled boxes represent neutralization of EV by antiserum pools A to H. The typing of a virus is based on combined neutralization of different antiserum pools. (Adapted from Melnick et al., 1979.)

information of a virus represents its ultimate characterization. All important information about a virus could potentially be obtained directly by PCR in conjunction with nucleic acid sequencing if all the molecular correlates of viral phenotypic determinants were understood. At present, however, the genetic locations for many properties of the virus remain uncertain. Nevertheless, it is possible at present to use sequence information to assign an EV isolate to a particular serotype (Caro et al., 2001; Casas et al., 2001; Norder et al., 2001; Oberste et al., 1999b; Oberste et al., 1999c; Oberste and Pallansch, 2005). The most common molecular typing system is based on reverse transcription (RT)-PCR and nucleotide sequencing of a portion of the genomic region encoding VP1. The serotype of an unknown isolate is inferred by comparison of the partial VP1 sequence with a database containing VP1 sequences for the prototype and variant strains of all human EV serotypes (Oberste and Pallansch, 2005). Using this approach, strains of homologous serotypes can be easily discriminated from heterologous serotypes and new serotypes can be identified. This method can greatly reduce the time required to type an EV isolate and can be used to type isolates that are difficult or impossible to type using standard immunologic reagents (Oberste et al., 2000). The technique is also useful to rapidly determine whether viruses isolated during an outbreak are epidemiologically related.

Serologic Diagnosis

Serologic diagnosis of EV infection can be made by comparing antibody titers in acute- and convalescent-phase (paired) serum specimens. If an EV isolation and identification have been made, paired serum specimens can be tested against the isolate. If not, it still may be possible to establish a likely EV infection if a particular serotype is suspected. In this case, a reference strain maintained in the laboratory can be used in a neutralization test. In general, however, EV serodiagnosis is more relevant to epidemiologic studies than to clinical diagnosis. The most basic serologic test is that of neutralization in cell culture. The mechanics of the assay are similar to the test used for identification of isolates, except that a known virus is mixed with serial dilutions of antisera from a patient (Weber et al., 1994). Other, less widely accepted serologic methods exist, all with particular strengths and weaknesses, including plaque reduction neutralization, complement fixation, passive hemagglutination, hemagglutination inhibition, and immunofluorescence (Melnick et al., 1979).

A fourfold or greater rise in type-specific neutralizing antibody titer is considered diagnostic of recent infection. Antibody, however, may already be present at the time the original specimen is obtained because of the extended incubation period and prodromal period of many enteroviral illnesses, which complicates interpretation of results.

Many serologic studies rely on the detection of IgM antibody as evidence for recent EV infection, and this is now widely used as an alternative to the neutralization and complement-fixation test. Several groups have developed an enzyme-linked immunosorbent assay (ELISA) for EV-specific IgM (Bell et al., 1986; Glimaker et al., 1992; Hodgson et al., 1995; Terletskaia-Ladwig et al., 2000). These tests have been found positive for nearly 90% of culture-confirmed CVB infections and can be performed rapidly. The ELISA has been successfully applied for epidemiologic investigations of outbreaks (Goldwater, 1995) as well as for specific diagnostic use (Day et al., 1989; Nibbeling et al., 1994; Wang et al., 2004). In most cases, the IgM ELISA test is not completely serotype specific. Depending on the configuration and sensitivity of the test, from 10% to nearly 70% of serum samples show a heterotypic response caused by other EV infections. This heterotypic response has been exploited to measure broadly reactive antibody, and the assay has been used to detect EV infection generically (Boman et al., 1992; Swanink et al., 1993). In attempting to characterize the exact nature of the response using different antigens, it is clear that the human immune response to EV infection includes antibodies that react with both serotype-specific epitopes and shared epitopes (Frisk et al., 1989). Despite this problem, which is inherently biologic, there is a fairly high concordance of results between assays of different configurations (Hodgson et al., 1995). In summary, the IgM assays that are generally used in epidemiologic studies have very good sensitivity and appear to be very specific for EV infection; however, these assays detect heterotypic antibodies resulting from other EV infections and, therefore, cannot be considered strictly serotype specific. A positive result with either the neutralization test or IgM ELISA indicates a recent viral infection; however, the infecting serotype found with the IgM assay may not be the same one determined by the neutralization test.

Parechovirus Diagnostics

Despite their original classification, sequencing and PCR studies demonstrated that E22 and E23 were distinct from the EV, resulting in their reclassification as members of a new picornavirus genus, *Parechovirus* (Coller et al., 1990; Hyypiä et al., 1992; Stanway et al., 1994; Stanway et al., 2005). Since then, four additional human parechovirus serotypes have been identified (Al-Sunaidi et al., 2007; Baumgarte et al., 2008; Benschop et al., 2006a; Ito et al., 2004; Watanabe et al., 2007a). Like the EV, human parechoviruses were traditionally detected and identified by virus isolation and antigenic typing (Lim and Benyesh-Melnick, 1960). By these methods, human parechovirus 1 (HPeV1; formerly E22) consistently accounted for 2 to 4% of EV reported to the CDC from 1975 to 1995 (Khetsuriani et al., 2006). RT-PCR began to supplant virus culture as the method of choice for EV detection in clinical diagnostic laboratories in the mid-1990s. Since that time, the number of HPeV1 reports has declined to under 1%, probably because HPeV-containing specimens are usually reported as EV PCR negative and not further characterized.

More recently, a number of investigators have developed conventional and real-time PCR assays to detect HPeVs (Baumgarte et al., 2008; Benschop et al., 2008; Benschop et al., 2006b; Corless et al., 2002; de Vries et al., 2008; Jokela et al., 2005; Joki-Korpela and Hyypia, 1998; Legay et al., 2002; Noordhoek et al., 2008; Oberste et al., 1999d; Read et al., 1997; Shimizu et al., 1995). These methods target conserved sites in the 5′ NTR that are analogous to those targeted by EV-specific PCR assays. The HPeV assays vary in level of validation; in particular, many have not been shown to detect the newly identified serotypes (Benschop et al., 2006b; Corless et al., 2002; Joki-Korpela and Hyypia, 1998; Legay et al., 2002; Oberste et al., 1999d; Read et al., 1997; Shimizu et al., 1995). Despite this possible limitation, a number of recent studies have applied molecular methods to the detection of HPeVs in patients with enteritis, respiratory illness, and neonatal sepsis-like syndrome (Al-Sunaidi et al., 2007; Baumgarte et al., 2008; Benschop et al., 2006b; Ito et al., 2004; Johansson et al., 2003; Johansson et al., 2002; Niklasson et al., 1999; Verboon-Maciolek et al., 2008; Watanabe et al., 2007a). As these methods are increasingly integrated into the diagnostic routine of clinical and reference laboratories, a better estimate will emerge of the burden of disease attributable to this group of picornaviruses.

PREVENTION AND THERAPY

Hygienic Prevention

No vaccines are available for nonpolio EV. General preventive measures include enteric precautions and good personal hygiene. Life-threatening infection is most common in newborns, although persons with compromised immune systems are also at higher risk. Hospital staff can inadvertently carry the virus between patients or become infected themselves and spread the virus. Patients with suspected EV infection should be managed with enteric precautions. Patients and staff can be cohorted during outbreaks. During several newborn outbreaks in hospitals, neonatal nurseries were closed to new admissions (Chen et al., 2005).

Immunization

By the 1950s, two different approaches to the prevention of poliomyelitis by vaccination were developed. The first successful polio vaccine, IPV, was produced by Salk and Youngner in 1954 by formaldehyde inactivation of cell culture-propagated virus (Salk and Salk, 1977). This vaccine was completely noninfectious, yet following injection, it elicited an immune response that was protective against paralytic disease. During the same period, many laboratories sought to produce live, attenuated polio vaccines. The OPV strains of Sabin were licensed in 1961, and widespread mass immunization campaigns in the United States began in 1962 (Sabin, 1985). Both IPV and OPV contain three components, one for each immunologically distinct serotype of PV.

The vast majority of countries use OPV exclusively, but an increasing number of industrialized countries use only IPV (Heymann et al., 2006). A limited number use both in sequence. Recommended vaccination schedules vary among countries, and debate continues about the relative merits of the two vaccines. The immunogenic and protective efficacies of OPV show geographic variation, and multiple doses are often required in some of the least developed countries of the tropics. Whereas in North America and Europe, almost all infants seroconvert to all three types of PV with 3 doses of OPV, in tropical developing countries, only 72% and 65% of infants develop antibodies to type 1 and 3 PV, respectively, in response to 3 doses of OPV, and additional doses are necessary to produce equivalent seroconversion rates (Patriarca et al., 1991).

Nevertheless, polio has been eliminated from the Americas, Europe, and the Western Pacific region, as well as tropical countries in South Asia, such as Bangladesh and Indonesia, using OPV exclusively. The reason for low seroconversion rates is not fully known, although recent studies strongly

implicate the presence of diarrheal disease at the time of OPV administration as an important factor.

While OPV is an extremely safe vaccine, it has been found to induce paralytic myelitis in a few instances. In primary vaccinees, vaccine virus-associated paralysis occurs at a rate of less than 1 in 500,000 (Alexander et al., 2004). The probability of vaccine-induced paralysis is higher in hypogammaglobulinemic children given OPV and in nonimmune adults in close contact with a recently immunized infant (Centers for Disease Control and Prevention, 2000). In tropical regions, where the risk of poliomyelitis due to wild PV is 1 in 200 to 1,000, the paralytic risk of OPV was not an important concern (Andrus et al., 1995; Kohler et al., 2002).

The contemporary IPV is highly immunogenic, and primary immunization consists of a minimum of 2 doses 8 weeks apart or an optimum of 3 doses, preferably at 8-week intervals (Dayan et al., 2007). When given as 2 doses, the first dose should be at or after 8 weeks of age; the 3-dose regimen may start at 6 weeks of age or thereafter. The effect of age and dose interval on seroconversion is under active investigation. A booster dose is recommended during the second year of life and around 5 years of age, to achieve a total of 4 doses, and this should suffice for long-term protection. Several IPV-containing combined vaccines are currently available to avoid the need for separate injections.

Polio Eradication

The epidemiology of PV infection has been radically altered by the widespread use of both IPV and OPV. The recent activities of the Polio Eradication Initiative have eliminated endemic PV from most of the world (Centers for Disease Control and Prevention, 2007a). Since 1988, poliomyelitis from wild PV has declined dramatically and in the year 2008 remained endemic to only four countries in Africa, the Middle East, and Southern Asia (Fig. 8). Three regions of the world—the Americas, the Western Pacific, and Europe—have been certified to be free of endemic PV transmission (Centers for Disease Control and Prevention, 2002). It is also highly likely that one of the three serotypes of wild PV (type 2) has already been eliminated from the entire world (Centers for Disease Control and Prevention, 2001). The only remaining wild type 2 viruses are now found in laboratories and vaccine manufacturing facilities. The eradication goal is attainable because humans are the only known reservoir for PV. The current global eradication program launched by WHO relies exclusively on OPV for mass vaccination. For developing countries, WHO recommends three routine doses of OPV be given at 6, 10, and 14 weeks of age, with an additional dose at birth in regions of endemicity where exposure of very young infants to wild virus can be expected (Sutter and Maher, 2006).

To achieve high vaccine coverage in all regions of the world, the Poliovirus Eradication Initiative also relies on supplemental immunization campaigns and aggressive investigation of all suspected cases of AFP to identify wild PV circulation (Deshpande et al., 2005). To ensure that no case of poliomyelitis is missed, every case of AFP in every location in every country must be detected through clinical surveillance and investigated virologically. Two stool samples should be collected on consecutive days, within 2 weeks after the onset of paralysis, and processed for virus isolation.

Any area with endemic wild PV can serve as a reservoir for reintroduction of PV to areas that have no endemic PV circulation (Centers for Disease Control and Prevention, 2006). In addition to several documented long-range importations over the past 25 years, wild PV recently spread from a reservoir of endemicity in Nigeria to cause cases in 18 additional countries during 2002 to 2005 and reestablish virus circulation in six of these countries. The frequency and ease of international travel probably results in frequent introduction of wild PV into all regions of the world. High rates of polio vaccine coverage are necessary to prevent poliomyelitis epidemics.

At least six poliomyelitis outbreaks have been associated with circulating vaccine-derived PV: on the island of Hispaniola in the Americas in 2000 to 2001, in the Philippines in 2001, in Madagascar in 2002, in China in 2004, in Nigeria in 2006 to 2007, and in Egypt from at least 1988 to 1993 (Centers for Disease Control and Prevention, 2007b; *Lancet*, 2007; Kew et al., 2002; Liang et al., 2006; Rousset et al., 2003; Shimizu et al., 2004a; Yang et al., 2003). The outbreak strains were unusual because their capsid sequences were derived from OPV. These viruses had recovered the capacity to cause paralytic poliomyelitis in humans and to be transmitted efficiently among human populations (Kew et al., 2005). Intense investigations suggest that circulation of vaccine-derived virus is a rare event, occurring only in populations with low immunization rates and high population densities. The recent discovery of circulating vaccine-derived PVs has created urgency in planning a comprehensive posteradication immunization strategy and emphasizes the fact that the risk of polio will not be eliminated until OPV vaccination stops. As a consequence, the eradication effort will need to address issues of future vaccination policy, the containment of laboratory and vaccine production strains, and a coordinated strategy to achieve the cessation of OPV immunization as vital parts of the eradication effort following the successful elimination of wild virus circulation (Heymann et al., 2006).

Therapy

Since no antiviral therapy is presently available for EV infections, treatment is directed toward alleviating symptoms. Treatment of acute poliomyelitis consists principally of supportive therapy and reduced physical activity. Mechanical ventilation is sometimes required in severe cases. Treatment of enteroviral meningitis is also supportive, and the course of illness is nearly always benign, usually lasting less than a week (Lee and Davies, 2007).

A number of specific antiviral compounds have been developed to target enteroviral proteins and steps in the virus' life cycle. Pleconaril or VP 63843 is a more recently developed pocket-binding compound with a broad in vitro inhibitory activity against 95% of the 215 nonpolio EV that were tested (Pevear et al., 1999). These drugs bind a hydrophobic site near the surface of the virion called the pocket (Grant et al., 1994), which lies in the floor of the canyon where the virion binds to the cellular receptor. By binding to the pocket, these compounds are believed to interfere with viral attachment and uncoating. A randomized, double-blind study involving the administration of Pleconaril following a challenge with CVA21 showed significant decreases in viral shedding in nasal secretions, nasal mucus production, and total respiratory illness symptom scores in patients treated with Pleconaril compared with subjects treated with placebo (Schiff and Sherwood, 2000).

Immunodeficient patients with persistent EV infections, including PV infections, represent a particular challenge to effective treatment. In patients with agammaglobulinemia, chronic EV infections have been treated with gamma globulin, and in some cases, this has controlled the infection (Rotbart and Hayden, 2000; Sawyer, 1999). Although intravenous

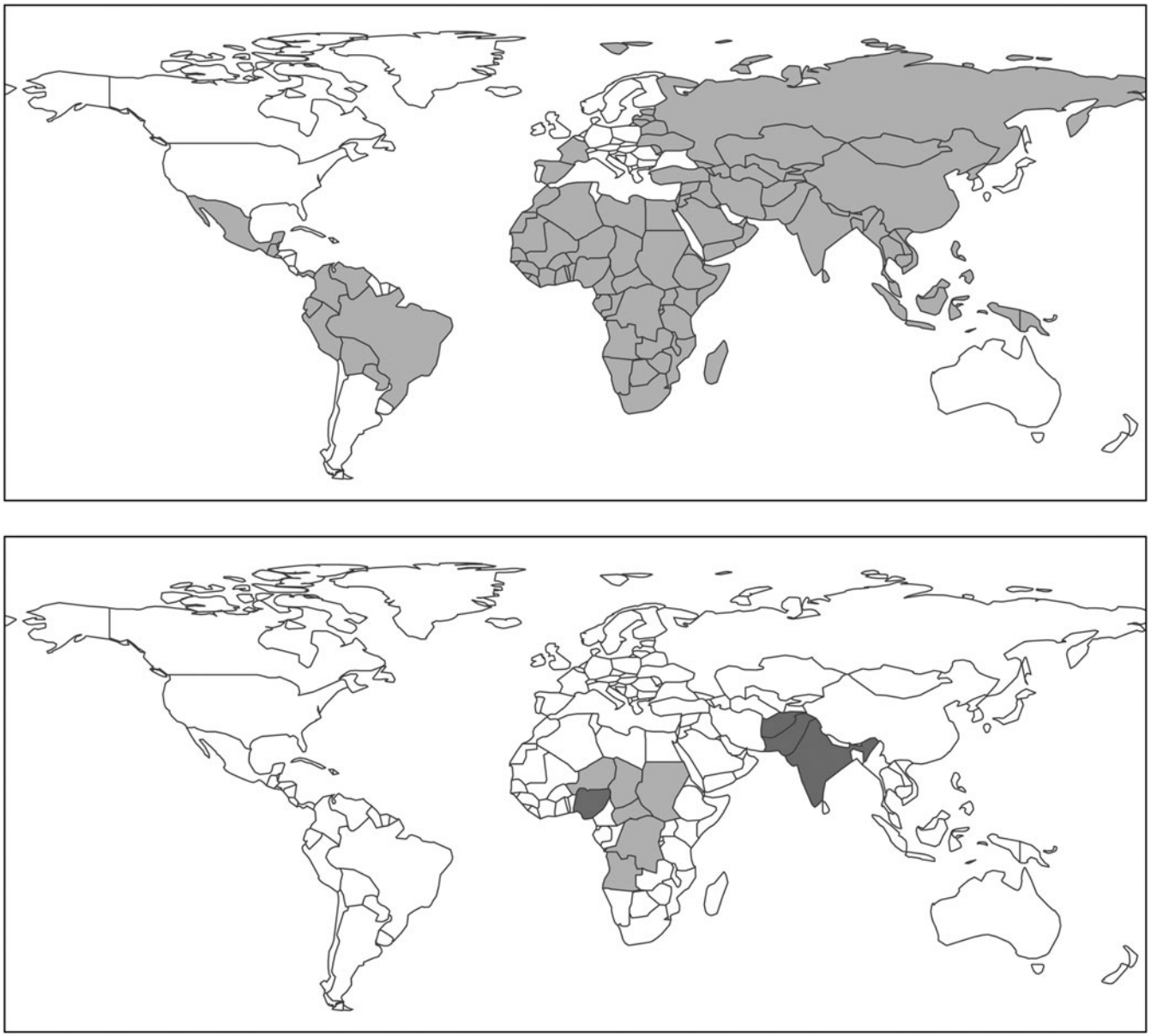

FIGURE 8 Global distribution of wild PV and progress in eradication. The maps indicate (shading) which countries had endemic wild PV circulation in 1988 (top) and 2007 (bottom). In 2007, several countries (light shading) that were polio free for at least 3 years had virus circulation following importations of wild PV and circulation was reestablished for a period of at least 12 months.

immunoglobulin (IVIG) may protect these patients from poliomyelitis and may appear to stabilize and improve some of the infections, the disease may progress. Many of these infections, however, spontaneously cease (McKinney et al., 1987; Misbah et al., 1992). Use of gamma globulin in other clinical illness has not been systematically evaluated. Pilot studies have been conducted administering IVIG in neonates suspected of having enteroviral infection (Abzug et al., 1995). Pooled immunoglobulin delivered intravenously or via a shunt into the spinal fluid also has been used in agammaglobulinemic patients with chronic encephalitis and meningitis associated with nonpolio EV (O'Neil et al., 1988). In some cases, efficacy may be limited by inadequate amounts of the relevant antibody in the immunoglobulin pool (e.g., if the infection involves an unusual and rare EV serotype) as well as problems in the delivery of adequate levels of antibody to the infected cells. One report documented the failure to clear persistent PV excretion despite treatment with IVIG, breast milk, and ribavirin (MacLennan et al., 2004).

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Rotavirus, Caliciviruses, Astroviruses, Enteric Adenoviruses, and Other Viruses Causing Acute Gastroenteritis†

TIBOR FARKAS AND XI JIANG

19

Acute gastroenteritis is an important cause of morbidity and mortality worldwide, particularly among young children living in developing countries. Among the several causes of gastroenteritis (chemical, bacterial, and parasitic), viral pathogens play an important role. Since the discovery of the Norwalk virus, the first viral agent linked to acute gastroenteritis in humans, in the early 1970s (Kapikian et al., 1972), a number of other viral agents associated with gastroenteritis in children and adults have been identified (Fig. 1). The inability or difficulty in cultivating most of the gastroenteritis viruses had a negative impact on research, especially on the development of immunological diagnostic methods for the detection of most of these viruses, particularly for human caliciviruses. Research of human gastroenteritis viruses has benefited from studies on similar animal viruses, such as animal rotaviruses. The molecular cloning of many gastroenteritis viruses in the early 1990s and subsequent development of new diagnostic methods, such as PCR, reverse transcriptase PCR (RT-PCR), and enzyme immunnoassays, significantly impacted our knowledge of viral gastroenteritis. The role of four viral agents, rotaviruses, caliciviruses, astroviruses, and enteric adenoviruses, in causing gastroenteritis in humans is well established. Others, such as coronaviruses, toroviruses, picobirnaviruses, and the Aichi virus, also have been indicated as causes of gastroenteritis in humans, but their significance remains to be established. The recent successful introduction of two rotavirus vaccines, Rotarix (GlaxoSmithKline Biologicals, Rixensart, Belgium) and RotaTeq (Merck & Co., Inc., Whitehouse Station, NJ), has opened a new era in the prevention and control of viral gastroenteritis and hopefully will be followed by other vaccine or antiviral strategies to further improve the health and life of the global population.

ROTAVIRUSES

Viruses with a wheel-like appearance (rota = wheel in Latin) had been described from a number of animal species in the 1960s (Adams and Kraft, 1963; Malherbe and Harwin, 1963; Malherbe and Strickland-Cholmley, 1967; Mebus et al., 1969). Human rotaviruses were first detected in 1973 by thin-section electron microscope examination of duodenal biopsy specimens obtained from children with acute diarrhea (Bishop et al., 1973). Rotaviruses today are recognized as the most important cause of severe infantile gastroenteritis worldwide.

Biology

Rotaviruses are members of the *Rotavirus* genus within the *Reoviridae* family, which also contains 10 other genera. The rotavirus genome consists of 11 segments of double-stranded RNA, ranging in size from ~660 bp (segment 11) to ~3,300 bp (segment 1). Each genomic segment codes for one viral protein, with the exception of segment 11, which codes for both NSP5 and NSP6 (Fig. 2) (Mattion et al., 1991). Rotavirus proteins include 6 structural proteins (VP1 to VP4, VP6, and VP7) that appear on the mature viral particles and 6 nonstructural proteins (NSP1 to NSP6) that are expressed in infected cells and are important in viral genome replication, protein synthesis, viral capsid assembly, and maturation (Estes, 2001). One of the nonstructural proteins, NSP4 is a viral enterotoxin (Ball et al., 2005; Ball et al., 1996b; Estes and Morris, 1999).

Rotaviruses are nonenveloped, approximately 75 nm in diameter, and composed of three concentric protein shells: the outer shell, the inner shell, and the core. The core is composed of 120 copies of VP2 and contains 11 viral genomic segments, the transcription enzyme complex of VP1 (RNA dependent RNA polymerase), and VP3 (guanylyl and methyl transferase). The inner shell is made up of 260 VP6 trimers which are interrupted by 132 aqueous channels. The outer shell consists of 260 VP7 trimers and 60 projecting VP4 dimers (Prasad and Chiu, 1994; Shaw et al., 1993).

VP6 is the dominant immunogen of rotaviruses and accounts for over 50% of the total protein mass of the virion. Based on the immunological specificity of VP6, seven distinct rotavirus groups (A though G) have been identified (Kapikian et al., 2001; Saif and Jiang, 1994) from which three (A, B, and C) have been linked to disease in humans

†This chapter contains information presented in the *Clinical Virology Manual*, 3rd ed. (Matson et al., 2000).

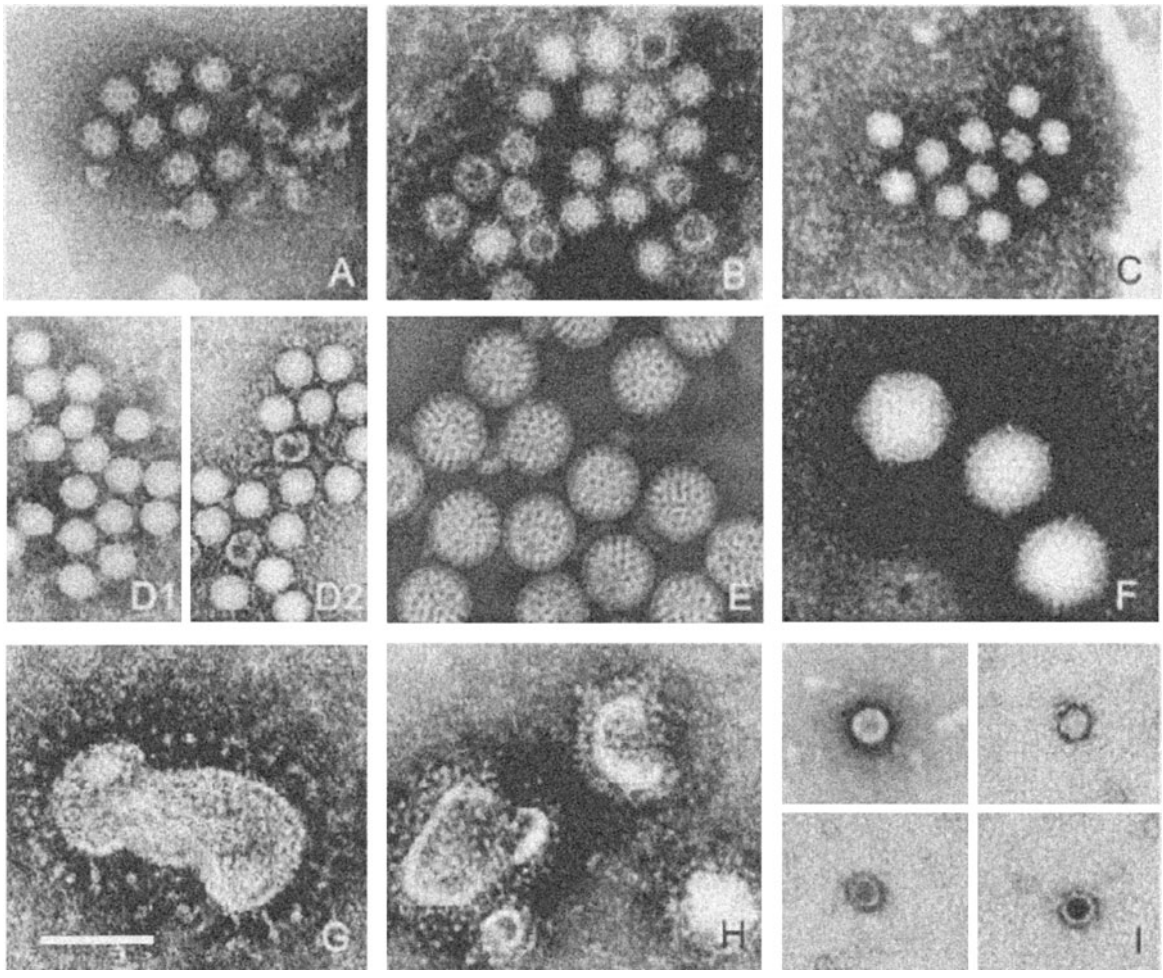

FIGURE 1 Electron micrographs of gastroenteritis viruses: (A) sapoviruses; (B) noroviruses; (C) astroviruses; (D) small round viruses; (E) rotaviruses; (F) adenoviruses; (G) coronaviruses; (H) torovirus-like particles; (I) picobirnaviruses. Bar = 100 nm.

(Table 1). Group A rotaviruses are the most common cause of infantile gastroenteritis and are the causative agent in more than 95% of rotavirus gastroenteritis in humans worldwide. Group B and C rotaviruses occasionally cause outbreaks and sporadic cases in adults and children and have been reported worldwide (Bridger et al., 1986; Cunliffe et al., 2001; Hamano et al., 1999; Hung et al., 1984; Jiang et al., 1995; Kang et al., 2005; Kelkar and Zade, 2004; Sanekata et al., 2003; Souza et al., 1998; Su et al., 1986).

Based on neutralization epitopes present on the outer shell proteins VP7 and VP4, group A rotaviruses are classified into G (VP7, glycoprotein) and P (VP4, protease sensitive) serotypes (Gorziglia et al., 1990; Hoshino and Kapikian, 1996). There are 14 VP7 (G) serotypes of rotaviruses and strains of animal and human origin may fall within the same serotypes (Gentsch et al., 1996). The G types have also been determined by sequencing or typing RT-PCR of gene segment 9 (VP7) and are referred to as G genotypes. Recently a new G genotype (G genotype 15) has been described in cattle (Rao et al., 2000), but serological typing of the new strain remains to be determined. Due to a lack of readily available typing antibodies for VP4 (P) serotypes, the classification of P types has mostly relied on genotyping. There are 27 P genotypes but only 14 P serotypes of VP4 described (Kapikian et al., 2001). Because of the genetic diversity of genome segment 4, the number of P genotypes is continuously expanding (Table 2) (Martella et al., 2007; Martella et al., 2006; McNeal et al., 2005; Rahman et al., 2005). A correlation between genotype and serotype has been observed for G types, but this correlation is less clear for P types, therefore numbers assigned for P serotypes and genotypes are different. According to the dual typing system, each virus is designated a P serotype (if available) and a P genotype (in square brackets) and a G type (genotype/serotype coinciding), e.g., P1A[8]G1. At least 10 G types (G1 to G6, G8 to G10, and G12) and 10 P types (P1A[8], P1B[4], P2A[6], P3[9], P4[10], P5A[3], P7[5], P8[11], P11[14], and P12[19]) have been associated with infection in humans (Kapikian et al., 2001).

Clinical Manifestation

Rotavirus infection can result in a wide spectrum of clinical manifestations ranging from asymptomatic infection to mild-to-severe malabsorptive diarrhea and/or mild-to-severe secretory diarrhea. It has an incubation period of 1 to 3 days (Davidson et al., 1975; Kapikian et al., 1983). Excretion of the virus in stool can precede the onset of clinical symptoms

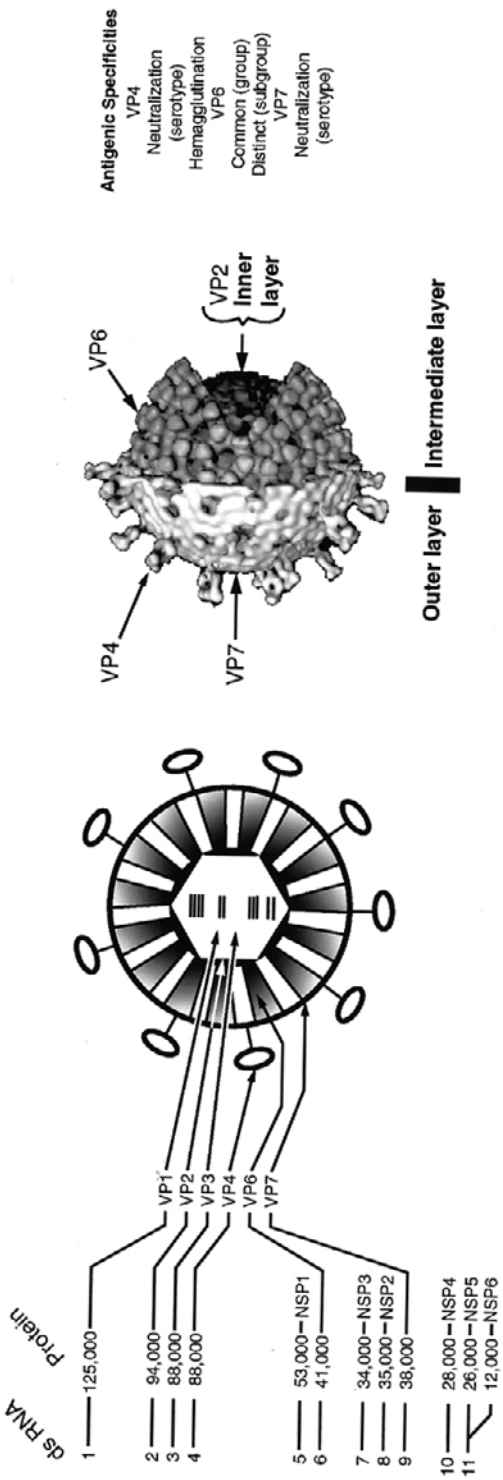

FIGURE 2 (Left) Schematic representation of rotavirus particle, gene segments, and encoded proteins. (Right) Surface representations of the three-dimensional structures of the outer layer of the complete particle ("Outer layer") and a particle ("Intermediate layer") in which the outer layer and a small triangular portions of the intermediate layer have been removed, exposing the inner layer. (Adapted from Kapikian et al., 2001.)

by several days and can continue for 8 to 10 days after symptoms end (Pickering et al., 1988). Rotavirus illness occurs in all age groups but is most common in infants from 6 months to 2 years of age. Asymptomatic rotavirus infections are common in neonates because of passively acquired maternal immunity, breastfeeding, and possible infection with less virulent strains (Bishop et al., 1983; Bryden et al., 1982; Chrystie et al., 1978; Matson, 1996; Rodriguez et al., 1982; Tam et al., 1990). This could also be explained by age-dependent changes in the intestine or decreased concentration of proteases (cleavage of VP4) in neonates. The reduced severity of rotavirus disease in older children and adults is possibly the result of acquired immune protection from previous rotavirus infections (Velazquez et al., 1996). In infants and young children, the most common clinical presentation is an abrupt onset of vomiting, followed by explosive watery diarrhea and moderate to high fever. The number of vomiting episodes and diarrhea determines the severity of the resulting dehydration. Dehydration most commonly is isotonic and may lead to metabolic acidosis and death (Carr et al., 1976; Echeverria et al., 1983; Rodriguez et al., 1977).

The mean duration of illness in immunocompetent hosts is 4 to 5 days, although mild infections can last 3 days or less and severe infections can last 10 days or more (Rodriguez et al., 1977). Rotavirus diarrhea is most commonly watery, occasionally with mucus. Bloody stools are rare and suggest the possibility of mixed infections if rotavirus is detected. Mixed rotavirus infections with other viral, bacterial, and parasitic enteropathogens account for up to 15% of diarrhea episodes in developing countries (Brandt et al., 1983; Prado and O'Ryan, 1994).

Respiratory tract symptoms, such as cough, pharyngitis, otitis media, and pneumonia, have been frequently associated with rotavirus infection, although this may represent concurrent winter respiratory virus infections (Goldwater et al., 1979; Lewis et al., 1979; Zheng et al., 1991). Rotavirus has been temporarily associated with a wide array of diseases, including intussusception, Reye's syndrome, encephalitis, aseptic meningitis, sudden infant death syndrome, inflammatory bowel disease, neonatal necrotizing enterocolitis, Kawasaki's syndrome, and myocarditis (Gregorio et al., 1997; Konno et al., 1978; Matsuno et al., 1983; Mulcahy et al., 1982; Nicolas et al., 1982; Rotbart et al., 1983; Salmi et al., 1978; Wong et al., 1984; Yolken and Murphy, 1982).

Chronic rotavirus infection can occur in immunodeficient children, although these infections are uncommon. Liver and/or kidney infection and damage in immunodeficient animals and humans have been reported (Gilger et al., 1992; Uhnoo et al., 1990a; Venuta et al., 2005). Infection of adults occurs often among those in close contact with young children and has been reported in travelers, military personnel, elderly persons in institutions, and hospitalized adults (Grimwood et al., 1983; Halvorsrud and Orstavik, 1980; Holzel et al., 1980; Hrdy, 1987; Kim et al., 1977; Marrie et al., 1982; Pickering et al., 1981; Vollet et al., 1979; von Bonsdorff et al., 1978). Infections in adults tend to be asymptomatic or to cause mild illness (Wenman et al., 1979).

TABLE 1 Host range of rotavirus antigenic groups

Group	Host(s) or reservoir(s)
A	Human, primate, horse, cow, pig, dog, cat, rabbit, mouse, bird
B	Human, cow, pig, sheep, rat
C	Human, pig, ferret
D	Chicken
E	Pig
F	Chicken
G	Chicken

Pathogenesis

The primary site of rotavirus infection is the mature enterocytes located in the mid- and upper villous epithelium (Davidson et al., 1975) of the upper small intestine, although lesions may extend to the distal ileum and colon (Greenberg et al., 1994; Phillipps, 1988). Once infected, the villous enterocyte is sloughed and the villus tips become denuded, resulting in an altered mucosal architecture which significantly reduces the surface area of the intestine. This is followed by a blastic response in the crypt cells, which tends to involve most of the affected villi. Diarrhea may result from decreased surface area, disruption in epithelial integrity, transient disaccharide deficiency, and/or altered countercurrent mechanisms. The mucosal damage can result in lactose intolerance that may prolong the duration of diarrhea (Kapikian et al., 2001).

The host range determinants of rotavirus infection are not well understood even though the role of several rotavirus proteins have been implicated (Bridger et al., 1998; Broome et al., 1993; Ciarlet et al., 1998b; Gombold and Ramig, 1986; Hoshino et al., 1995; Kojima et al., 1996; Taniguchi et al., 1994). VP3, VP4, VP7 and NSP4 were clearly associated with virulence in a pig model (Hoshino et al., 1995). Substitution of any of these genes of a virulent porcine rotavirus by the corresponding gene segment from a human rotavirus resulted in an asymptomatic infection. Rotavirus NSP4 was found to be the first viral enterotoxin, since it induces age-dependent diarrhea in mice by triggering calcium-dependent chloride and water secretion (Ball et al., 1996b). The diarrhea-inducing effect of NSP4 has been demonstrated for several group A rotaviruses, one group B rotavirus, and one group C rotavirus (Horie et al., 1999; Ishino et al., 2006; Mori et al., 2002; Rodriguez-Diaz et al., 2003; Sasaki et al., 2001; Zhang et al., 1998). The enterotoxin domain of NSP4 has been mapped to a region of amino acid positions 114 to 135 (Ball et al., 1996b). Inflammation caused by bacterial enterotoxins has been known to evoke fluid secretion by the activation of the enteric nervous system. In a mouse model, the role of the enteric nervous system in rotavirus-induced secretory responses also has been indicated (Lundgren et al., 2000). Whether this response is triggered by NSP4 remains to be elucidated.

TABLE 2 G and P types of group A rotaviruses[a]

Designation and distribution of G types			Designation and distribution of P types			
Type	Human	Animal species	Genotype	Serotype	Human	Animal species
1	Yes	Cow	1	6		Cow, monkey
2	Yes		2	5B		Monkey
3	Yes	Monkey, dog, cat, rabbit, mouse, pig	3	5	Yes	Monkey, dog, cat
4	Yes	Pig	4	1B	Yes	
5	Yes	Pig, horse	5	7		Cow
6	Yes	Cow	6	2A	Yes	
				2B		Pig
7	No	Chicken, turkey, pigeon, cow	7	9		Pig
8	Yes	Cow	8	1A	Yes	
9	Yes		9	3	Yes	Cat
10	Yes	Cow, sheep	10	4	Yes	
11		Pig	11	8	Yes	Cow
12	Yes		12	4		Horse
13		Horse	13		Yes	Pig
14		Horse	14	11	Yes	Rabbit
15		Cow	15			Sheep
			16	10		Mouse
			17			Cow, pigeon
			18			Horse
			19	12	Yes	Pig
			20	13		Mouse
			21			Cow
			22			Sheep
			23			Pig
			24			Monkey
			25		Yes	
			26			Pig
			27			Pig

[a]Modified from Kapikian et al., 2001.

Diverse mechanisms participate in the protective immune response against rotavirus infection and illness involving both humoral (specific for isotype, VP7, VP4, and other viral proteins) and cell-mediated immune mechanisms. The role of humoral immunity in protection against rotavirus infection or illness is well characterized. Although rotavirus can infect a child many times, second and subsequent infections are less likely than first infections to be symptomatic, and two rotavirus infections confer a complete protection against a third severe symptomatic infection (Velazquez et al., 1996). This protection against rotavirus infection and/or illness correlates with the presence and titer of either serum or mucosal antirotavirus antibodies (immunoglobulin M [IgM], IgG, IgA) and is mainly homotypic (Coulson et al., 1990; Davidson et al., 1983; Grimwood et al., 1988; Hjelt and Grauballe, 1990; Hjelt et al., 1987; Matson et al., 1993; O'Ryan et al., 1994; Riepenhoff-Talty et al., 1981; Xu et al., 2005). Reinfection is more likely to occur by a G type different from that causing the initial infection (Velazquez et al., 1996). Breastfeeding protects against rotavirus infections by decreasing exposure and providing anti-rotavirus IgA antibodies and the rotavirus-binding glycoprotein lactadherin (Gianino et al., 2002; Guerrero et al., 2004; Kvistgaard et al., 2004; Mastretta et al., 2002; Newburg et al., 1998).

The role of cell-mediated immunity in rotavirus infection also has been investigated mainly in animal models. Murine rotavirus-specific $CD8^+$ T cells are clearly involved in the resolution of primary rotavirus infection, since depletion of $CD8^+$ cells in B-cell-deficient mice prior to infection with murine rotavirus prevents resolution of the infection (Franco and Greenberg, 1995; Franco et al., 1997; McNeal et al., 1995). Also, adoptive transfer of $CD8^+$ T cells from mice previously infected with murine rotavirus was able to provide passive protection against rotavirus disease in the neonatal mouse model (Offit and Dudzik, 1990). Moreover, when mice were intranasally immunized with a chimeric VP6 protein and adjuvant, only $CD4^+$ cells were required to resolve challenge virus shedding (Choi et al., 1999; McNeal et al., 2002).

Epidemiology

Globally, each year approximately 111 million episodes of gastroenteritis requiring home care, 25 million clinic visits, 2 million hospitalizations, and 352,000 to 592,000 deaths in children less than 5 years of age can be linked to rotavirus infection. By age 5, almost every child will have at least one episode of rotavirus gastroenteritis, 1 in 5 will require a clinic visit, 1 in 65 will be hospitalized, and approximately 1 in 293 will die due to rotavirus disease (Parashar et al., 2003).

The incidence of rotavirus diarrhea is similar in children in both developed and developing countries; however, the disease is more often severe and with a greater rate of fatal infection in children in developing countries probably due to delayed medical care and higher rates of malnutrition. An estimated 1,205 children die from rotavirus disease each day, and 82% of these deaths occur in the poorest countries (Parashar et al., 2003). In the United States, rotavirus diarrhea is responsible for more than 600,000 clinic visits, 55,000 to 70,000 hospitalizations, and 20 to 60 deaths of children each year (Parashar and Glass, 2006). The rotavirus-associated medical and societal costs in the United States are estimated at $1 billion per year.

In temperate countries, rotavirus infections are most common during the autumn and winter seasons when it can be the causative agent for up to 70% of hospital admissions for diarrhea (Bartlett et al., 1988; Glass et al., 1996a; Konno et al., 1983; Koopmans and Brown, 1999). The reason for this seasonality is unknown. In tropical areas, rotavirus tends to circulate year-round without seasonality, but rotavirus disease is still more prevalent in the drier, cooler months (Cook et al., 1990).

Information on the epidemiology of rotavirus G and P types is available for most areas worldwide. The vast majority of rotavirus infections (~95%) are caused by G1, G2, G3, or G4 in association with the P1A[8] and P1B[4] serotypes (Gentsch et al., 1996; Koopmans and Van Asperen, 1999; Santos and Hoshino, 2005). Other G and P types also have been frequently isolated particularly in developing countries and are becoming prevalent, such as G9 strains, which have been found worldwide, sometimes representing a large fraction of the isolates (Gentsch et al., 1996; Marmash et al., 2007; Montenegro et al., 2007; Reidy et al., 2005). Several antigenic types may cocirculate in large urban areas, although one or two types usually predominate (Gentsch et al., 1996). Changes of predominant types are regional events and are probably limited by the extent of exposure and herd immunity. The appearance of new types suggests that reassortment between common and uncommon human and/or animal strains occurs, and in some locations, otherwise uncommon types can predominate (Gentsch et al., 1996; Palombo and Bishop, 1995).

Rotaviruses are transmitted from person to person by the fecal-oral route. Rapid spread via airborne droplets also has been suspected (Foster et al., 1980). As few as 100 virus particles are sufficient to cause infection, and at the peak of infection, as many as 10^{11} rotavirus particles per gram of stool are excreted (Nagayoshi et al., 1980; Vesikari et al., 1981). Rotaviruses are stable in the environment, and they may remain infectious for many months at ambient temperature (Abad et al., 1994; Keswick et al., 1983b; Tan and Schnagl, 1981). Contaminated water or food can be the source of rotavirus-associated outbreaks, although the majority of food- and water-related outbreaks are caused by noroviruses (Centers for Disease Control and Prevention, 2000; Hopkins et al., 1984; Mikami et al., 2004). Crowdedness is a significant risk factor for increased rotavirus transmission in families and child-care centers (Gurwith et al., 1983; Keswick et al., 1983a).

Diagnosis

Many methods can be used for detection and typing of rotaviruses from stool samples, including enzyme-linked immunosorbent assay (ELISA), latex agglutination, RT-PCR, gel electrophoresis of the viral genomic RNA, electron microscopy (EM), immune EM (IEM), and cell culture. ELISA is the most commonly used in the clinical diagnostic laboratories. The latex agglutination test can provide very rapid results, which is particularly useful in laboratories in remote areas without sufficient equipment for other tests. The RT-PCR methods are highly sensitive and are particularly useful for genotyping (G and P types) of rotaviruses for molecular epidemiology studies in the research laboratories.

Stool specimens collected within the first few days of illness contain the highest concentration of virus. Specimens can be collected as bulk stool from diapers or as rectal swabs. For optimal detection, stool specimens should be tested immediately or, if necessary, they can be kept at 4°C for a few days. For longer storage, samples should be frozen at –70°C, but repeated freezing and thawing should be avoided.

Cell Culture

Cell culture is not used for the diagnosis of rotavirus disease, since human rotaviruses are difficult to cultivate from

clinical specimens. A variety of cell lines have been tested and utilized for rotavirus isolation, including MDBK, PK-15, BSC-1, LLC-MK2, BGM, CV-1, MA104, CaCo-2, and HRT-29. Incorporation of proteases (trypsin) into the culture medium is necessary (Birch et al., 1983; Hasegawa et al., 1982; Kutsuzawa et al., 1982; Sato et al., 1981; Wyatt et al., 1980). Rotavirus cell cultures are particularly useful in typing of rotaviruses based on a neutralization test with type-specific antibodies.

Serology

Neutralization assays have been used to determine antibody responses to specific rotavirus serotypes (Kapikian et al., 2001; Ward et al., 2006). Alternatively, epitope-blocking assays utilizing monoclonal antibodies (MAbs) can be used to measure specific antibody responses to specific epitopes, such as those that are G type specific (Matson et al., 1992; Shaw et al., 1985). Group-specific antibodies (IgM, IgG, IgA) can be measured by antigen detection ELISAs using any group A rotavirus as the test antigen. About one-third of rotavirus infections in infants are detected only by serological response because virus excretion is below virus detection assay limits (Matson et al., 1993; Velazquez et al., 1996).

Immunoassays

ELISAs and latex agglutination assays are simple, sensitive and rapid assays and are currently the most widely used methods for diagnosis of rotavirus infection and illness in general clinical laboratories. Many of these assays are commercially available with high specificity and sensitivity (Dennehy et al., 1999; Eing et al., 2001; Lipson et al., 2001; Raboni et al., 2002; Thomas et al., 1988). MAbs to a wide variety of rotavirus epitopes have been developed. G-typing MAbs have been widely used as research tools in epidemiologic studies (Cascio et al., 2001; Gentsch et al., 1996; O'Ryan et al., 1990; Raj et al., 1992; Szucs et al., 1995). P-typing MAbs are more difficult to generate and have limited applicability. Due to antigenic changes over time, more and more clinical strains are nontypable using the MAbs generated earlier and RT-PCR-based genotyping methods are more commonly used.

Genomic Analysis

RT-PCR using common or type-specific primers is 10 to 100 times more sensitive than ELISA for the detection of rotaviruses from clinical specimens, but RT-PCR is not commonly used for diagnosis in general clinical diagnostic laboratories due to the requirement of sophisticated equipment, well-trained personnel, and longer turnaround time than the antigen detection methods. RT-PCR followed by sequencing or with type-specific primers targeting gene segments 4 (VP4) or 9 (VP7), which amplify fragments of different lengths, are widely used for G or P genotyping (Gentsch et al., 1992; Gouvea et al., 1990; Green et al., 1988; Iturriza-Gomara et al., 2004; Nakagomi et al., 1991).

EM and IEM

Rotaviruses are readily recognized in stool specimens by direct EM because of the large number of virions in specimens and their characteristic morphology and size (Hammond et al., 1982). IEM, which is based on the formation of virus-antibody aggregates, can increase the sensitivity of EM by 100-fold; however, particles are often covered with antibody, which can mask virus structure (Brandt et al., 1981; Gerna et al., 1989; Wu et al., 1990). Coating the grid with specific antibody before applying the sample (solid-phase IEM) avoids this problem.

Electropherotyping

Electropherotyping is the assessment of the migration pattern of the 11 rotavirus genomic segments. The viral RNA is extracted directly from stool specimens and then subjected to electrophoresis, followed by detection with silver staining (Herring et al., 1982). This method is as sensitive as EM and was most widely used for diagnosis of rotavirus before immunoassays were developed. It is still used in epidemiology studies where the appearance of an unusual electropherotype could denote a novel strain or group of rotavirus (Garbag-Chenon et al., 1985). Electropherotyping also has limitations because many electropherotypes exist within one G and/or P type and similar electropherotypes may occur in different antigenic types (Gerna et al., 1987; O'Ryan et al., 1990; Pipittajan et al., 1991). Electropherotyping is the only widely available method for identifying non-group A rotaviruses (Kuzuya et al., 1996). An EM-positive sample that does not react in commercial antigen detection assays can be assigned to group B to F by the markedly different electropherotypes of non-group A rotaviruses. There is no established classification based on electropherotypes.

Control and Prevention

There is no effective antiviral agent against rotaviruses. Oral or intravenous rehydration therapy is a common supportive treatment of severe rotavirus diarrhea. The significant disease burden of rotaviruses has necessitated the development of a vaccine against rotavirus disease for quite a long time. Unfortunately, the first licensed rotavirus vaccine (Rotashield; Wyeth-Lederle), which was commercially available in 1998 in the United States, was withdrawn in 1999 due to the concern that it increased the incidence of intussusception (Centers for Disease Control and Prevention, 1999a, 1999b, 2004). A lamb rotavirus-based, live, attenuated vaccine (LLR; Lanzhou Institute of Biological Products) was licensed in China in 2000, but little information is available about the safety and usefulness of this vaccine. A promising rotavirus vaccine (Rotarix; GlaxoSmithKline) based on an attenuated single human strain has been licensed in Mexico since 2004 and was later licensed in over 35 countries and the European Union (Glass et al., 2006). Rotarix has been listed in the national immunization programs of Brazil, El Salvador, Mexico, Panama, and Venezuela. The newest rotavirus vaccine, developed by Merck (RotaTeq; Merck & Co., Inc.), is also a live, attenuated vaccine containing five human-bovine (WC3) reassortants. This was licensed in early 2006 in the United States (Glass et al., 2006). It is believed that worldwide marketing and use of these rotavirus vaccines will significantly reduce the disease burden of rotavirus gastroenteritis and improve the health of children, especially in resource-poor countries.

Several other vaccine approaches have also been investigated for rotaviruses, such as subunit vaccines of virus-like particles expressed in baculovirus (Ciarlet et al., 1998a; Fernandez et al., 1996; Jiang et al., 1999a), rotavirus proteins expressed in bacteria (Choi et al., 2004), DNA vaccines (Choi et al., 1997; Herrmann et al., 1996), or killed rotavirus vaccines (Coffin et al., 1995). At present, none of these has yet entered clinical trials.

CALICIVIRUSES

Caliciviruses are single-stranded, positive-sense RNA viruses with a broad host range and disease manifestation, such as respiratory disease in cats (feline calicivirus), vesicular disease in swine (vesicular exanthema of swine virus), and

hemorrhagic disease in rabbits (rabbit hemorrhagic disease virus). In humans, caliciviruses are a major cause of sporadic and epidemic acute gastroenteritis worldwide.

Biology

The *Caliciviridae* family consists of four genera, *Vesivirus*, *Lagovirus*, *Norovirus*, and *Sapovirus*. Recently, analysis of the complete genomic sequence of a bovine calicivirus (Newbury agent 1) revealed that it represents a new, fifth genus with a proposed name "Becovirus" (Oliver et al., 2006), and a possible sixth genus is represented by a unique calicivirus that was isolated from rhesus monkeys (T. Farkas, personal communication) (Fig. 3). The name calicivirus (calix = cup in Latin) refers to the cup-shaped depressions that are seen on the surface of virions under an EM.

Members of the *Norovirus* and *Sapovirus* genera are causative agents of acute gastroenteritis in humans and are often referred to as human caliciviruses (HuCV); however, caliciviruses of animal origin belonging to these two genera have also been isolated (Guo et al., 1999; Guo et al., 2001a; Liu et al., 1999; Sugieda et al., 1998). These two genera, previously called "Norwalk-like viruses" and "Sapporo-like viruses," have been named after their prototype strains, the Norwalk virus (now *Norovirus*) (Kapikian et al., 1972) and Sapporo virus (now *Sapovirus*) (Chiba et al., 1979), respectively. Based on phylogenetic analysis of the viral genome and mainly deduced capsid protein amino acid sequences, HuCVs are further classified within each genus into genogroups and within genogroups into genetic clusters. Norovirus genogroups (GG) I, II, and IV contain the human isolates, GG III the bovine strains, and GG V the recently discovered murine noroviruses (Karst et al., 2003; Zheng et al., 2006). There are over 25 established norovirus genetic types within the five norovirus genogroups (Zheng et al., 2006). According to this classification the prototype, Norwalk virus, represents GG I/1 (genogroup/genotype). Similarly, sapoviruses can be classified into at least 10 genetic types within five genogroups, of which GG I, II, IV, and V contain human isolates (Farkas et al., 2004). New genetic types are continually being identified within both genera, indicating an extensive genetic and probably antigenic diversity of these viruses.

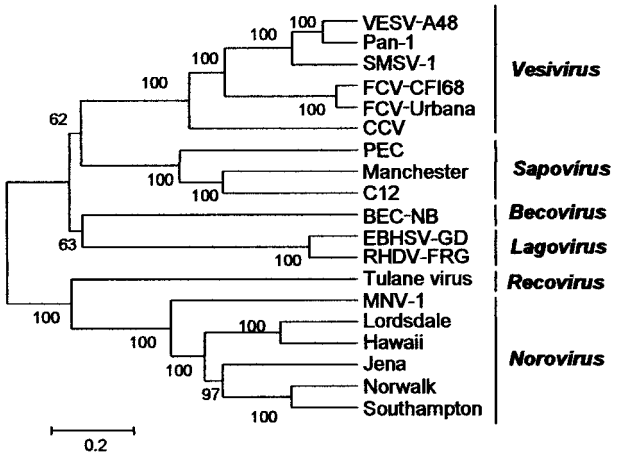

FIGURE 3 Classification of *Caliciviridae*. Phylogenetic tree based on deduced capsid amino acid sequences. The tree was constructed by the unweighted-pair group method using average linkages clustering method (MEGA v3.1). VESV, vesicular exanthema of swine virus; Pan-1, primate calicivirus; SMSV, San Miguel sea lion virus; FCV, feline calicivirus; CCV, canine calicivirus; PEC, porcine enteric calicivirus; EBHSV, European brown hare syndrome virus; RHDV, rabbit hemorrhagic disease virus; MNV-1, murine norovirus. *Becovirus* (bovine enteric calicivirus) is represented by the Nebraska strain (Smiley et al., 2002; Oliver et al., 2006). The *Recovirus* (rhesus enteric calicivirus) genus name has been proposed for Tulane virus (Farkas et al., 2008).

The antigenic relationship among different HuCV genetic types is not well characterized; however, limited studies that were based on recombinant virus-like particles and antibodies raised against them in laboratory animals suggest a strong correlation between capsid-based genotypes and antigenic types (Farkas et al., 2006; Green et al., 1995; Hansman et al., 2005a; Hansman et al., 2005b; Jiang et al., 1996; Jiang et al., 2002). Because of the lack of a cell culture system, serotyping of HuCVs has not yet been achieved.

Caliciviruses are small (30 to 38 nm), round, nonenveloped viruses. Two morphologic types have been described: "typical caliciviruses" (*Sapovirus*, *Vesivirus*, and *Lagovirus*), with a "Star of David" appearance, and "small, round structured viruses" (*Norovirus*), which have a smoother surface structure (Green et al., 2001). This morphologic distinction is not always clinically useful because particle morphology is frequently indistinct in clinical samples. The viral genome is ~7.5 kb long (Jiang et al., 1990; Lambden et al., 1993), which in the case of *Norovirus* and *Vesivirus*, is organized into three open reading fames (ORF), with ORF1 encoding the nonstructural proteins, ORF2 the capsid protein, and ORF3 a minor structural protein (Glass et al., 2000; Jiang et al., 1993b). In the case of the *Sapovirus* and *Lagovirus* genomes, the nonstructural and capsid protein-encoding sequences are fused into one large ORF (Liu et al., 1995; Rasschaert et al., 1995). An ~2.5-kb subgenomic RNA is also encapsidated in mature virions and is believed to be the transcript for capsid protein synthesis (Dunham et al., 1998; Herbert et al., 1996; Meyers et al., 1991). Caliciviruses encode a single capsid protein of ~60 kDa that self-assembles into virus-like particles when expressed in baculovirus, eukaryotic cells, or yeast (Harrington et al., 2002; Jiang et al., 1990; Jiang et al., 1992; Jiang et al., 1999d; Xia et al., 2007). A smaller cleavage product of the capsid protein (~30 kDa), consisting of the P domain that is released from a trypsin cleavage site in the hinge-P domain junction, also has been found in stools of patients and in baculovirus expression cultures (Greenberg et al., 1981; Hardy et al., 1995; Jiang et al., 1992).

Clinical Manifestation

The main clinical features of HuCV infection include nausea, abdominal cramps, vomiting, diarrhea, and fever. The diarrhea is often watery, without mucus, blood, or leukocytes. Vomiting is more frequent in children than in adults. The clinical features of sapovirus and norovirus gastroenteritis are similar, but sapoviruses more frequently cause disease in infants and toddlers than in school-aged children, while noroviruses are frequent in all age groups (Pang et al., 1999; Pang et al., 2001; Rockx et al., 2002). The incubation period has a mean of 24 to 48 hours, with a range of 4 to 77 hours (Kaplan et al., 1982). The duration of illness is usually 12 to 48 hours, after which it resolves. Maximal excretion of the virus in feces can be detected at the onset of clinical symptoms and shortly thereafter; however, virus shedding can continue for up to 2 to 3 weeks after clinical symptoms resolved, which has important implications in disease control (Graham et al., 1994; Murata et al., 2007; Rockx et al., 2002). Prolonged shedding of noroviruses in immunocom-

promized patients has been described previously (Gallimore et al., 2004; Nilsson et al., 2003; Simon et al., 2006).

Pathogenesis

The primary site of HuCV replication is believed to be the proximal part of the small intestine. Jejunal biopsy specimens obtained in volunteer challenge studies revealed histologic changes, such as broadening and blunting of the villi, crypt cell hyperplasia, mononuclear cell infiltration of the jejunal mucosa, and cytoplasmic vacuolization (Agus et al., 1973; Dolin et al., 1976; Schreiber et al., 1973, 1974). The gastric and colonic mucosae remained histologically normal. These abnormal findings persisited for at least 4 days after clinical symptoms ceased; however, the virus could not be detected in abnormal mucosal cells. Pathologic examination of patients following sapovirus infection is lacking. A gnotobiotic pig model using porcine enteric calicivirus, a sapovirus, revealed similar histopathologic changes as seen in biopsy specimens of human volunteers in the duodenum and jejunum of infected animals (Flynn et al., 1988; Guo et al., 2001b). No virus replication was observed in the colon or extraintestinal tissues; however, a low level of viremia could be detected in acute-phase sera. In a recent report, infection of gnotobiotic pigs with a GG II human norovirus isolate resulted in mild diarrhea in over 70% of the animals, and immunoflourescence microscopy revealed patchy infections of duodenal and jejunal enterocytes, with only a few stained cells in the ileum (Cheetham et al., 2006).

Immunity following norovirus infection is believed to be short-lived and homotypic. Volunteers who became ill following challenge with the prototype Norwalk virus (GG I/1) were protected against rechallenge with the same virus 6 to 14 weeks later, but this immunity was not able to provide protection against rechallenge with the Hawaii virus (GG II/1) (Wyatt et al., 1974). Several other volunteer studies and natural outbreak investigations indicated the involvement of a nonimmunologic factor(s) in protection or resistance against norovirus infection (Baron et al., 1984; Blacklow et al., 1979; Cukor et al., 1982; Graham et al., 1994; Johnson et al., 1990).

Recently, one of these factors has been found to be the involvement of human histo-blood group antigens (HBGAs) in norovirus infection (Hutson et al., 2002; Lindesmith et al., 2003; Marionneau et al., 2002). Noroviruses bind to HBGAs in a specific manner, and the binding specificity varies among different noroviruses. Three major HBGA types, the ABO, Lewis, and secretor types, are involved in this interaction, and 8 receptor binding patterns have been identified (Harrington et al., 2004; Huang et al., 2003; Huang et al., 2005). The prototype Norwalk virus represents one binding pattern that recognizes type A and O antigens, less effectively recognizes type B antigen, and cannot bind to nonsecretor HBGAs. Volunteer studies with the Norwalk virus showed an excellent correlation between clinical infection and the binding pattern (Hennessy et al., 2003; Hutson et al., 2002; Lindesmith et al., 2003). This finding is highly significant and will impact norovirus research in many areas, although such correlation between host range variability and HBGA binding specificity for the remaining binding patterns remains to be demonstrated. Since the discovery of HBGA involvement in Norwalk virus binding in 2000, the study of norovirus receptor interaction rapidly advanced, and a detailed receptor binding interface on the norovirus capsid has been elucidated by mutagenesis and cocrystallization studies (Cao et al., 2007; Tan et al., 2003).

The discovery of animal caliciviruses that are genetically closely related to human strains and studies that indicated that human noroviruses bind HBGAs present in animal excretions or on the surface of animal tissues (Cheetham et al., 2007; Tian et al., 2005; Tian et al., 2007) raised questions about norovirus gastroenteritis as a zoonotic disease and the possible role of animals as reservoirs of human infections. Currently, there is no direct evidence for interspecies transmission of enteric caliciviruses.

Epidemiology

Noroviruses have been recognized as the most important causes of nonbacterial acute gastroenteritis for all ages in both developing and developed countries. The major public health concern of noroviruses is their ability to cause large outbreaks of gastroenteritis. Over 80% of the nonbacterial gastroenteritis outbreaks and >50% of all food-borne outbreaks of gastroenteritis are caused by noroviruses (Fankhauser et al., 1998; Lopman et al., 2003; Svraka et al., 2007). Such outbreaks mainly occur in closed or semiclosed communities (schools, summer camps, cruise ships, military ships, restaurants, and hospitals), and contaminated food or water is often indicated as the common source of infection (Baron et al., 1982; Fankhauser et al., 1998; Farkas et al., 2003; Kaplan et al., 1982; Le Guyader et al., 2006). The fecal-oral transmission route, the low infectious dose (<100 virus particles), and the high environmental stability of noroviruses are important features for the rapid spread of infection. Spread of noroviruses by aerosols generated from vomitus of infected individuals have also been suspected (Marks et al., 2000; Marks et al., 2003). Because of their ability to cause large outbreaks with incapacitation of the affected individuals, noroviruses are listed as category B agents by the National Institute of Allergy and Infectious Diseases Biodefense Research Agenda.

The importance of noroviruses in sporadic cases of pediatric gastroenteritis is also well documented. Noroviruses have been detected in up to 20% of diarrhea episodes that occurred at home or in patients that were seen in a physician's office setting (Borges et al., 2006; de Wit et al., 2001; Farkas et al., 2000; Pang et al., 2000; Pang et al., 1999). Even though the severity of norovirus diarrhea is believed to be relatively mild compared to that of rotaviruses, a high detection rate (≥30%) of noroviruses has been reported among children hospitalized for acute gastroenteritis (Chen et al., 2006; Colomba et al., 2006; Kirkwood and Bishop, 2001; Medici et al., 2004; Oh et al., 2003; Zintz et al., 2005), indicating a significant role of noroviruses in pediatric hospitalizations. Norovirus infections occur year-round, although they are more common in the winter season.

Epidemiologic patterns of sapovirus infections are similar to those of noroviruses, but probably due to persistent immunity developed following sapovirus infections, sapovirus gastroenteritis is mainly a pediatric disease. A few sapovirus gastroenteritis outbreaks among adults have been reported (Chiba et al., 1979; Johansson et al., 2005; Noel et al., 1997b). Sapoviruses have been detected in up to 10% of sporadic cases of gastroenteritis in children <5 years of age (Farkas et al., 2000; Koopmans et al., 2001; Pang et al., 2000; Phan et al., 2005a; Phan et al., 2007) and in up to 11% of specimens collected from children hospitalized for gastroenteritis (Guntapong et al., 2004; Hansman et al., 2004; Kirkwood et al., 2005; Simpson et al., 2003; Zintz et al., 2005).

Antibody prevalence studies suggest that almost every child possesses antibodies to both noroviruses and sapoviruses by 5 years of age, indicating mass circulation of these pathogens in communities worldwide (Fig. 4) (Farkas et al., 2006; Jing et al., 2000; Nakata et al., 1996; Smit et al., 1999).

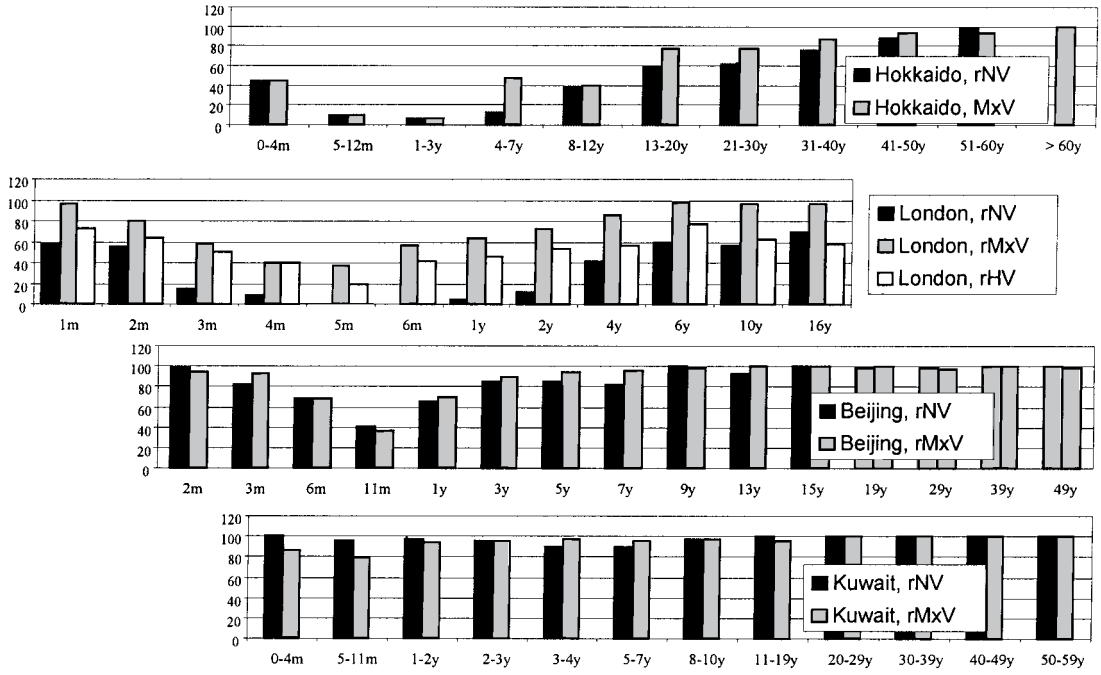

FIGURE 4 Antibody prevalence by age to noroviruses in Japan, United Kingdom, China, and Kuwait measured by antibody detection ELISAs utilizing recombinant norovirus virus-like particles (Jing et al., 2000). rNV, Norwalk (GGI/1); rMx, Mexico (GGII/3); rHV, Hawaii (GGII/1); m, months; y, years.

Diagnosis

Stool specimens collected from symptomatic patients are most likely to yield positive results for HuCV detection. Detection of noroviruses in vomitus has also been reported (Greenberg et al., 1979). A number of methods have been developed for the diagnosis of HuCVs, including direct visualization of virions (EM and IEM), detection of viral antigens (ELISA), and amplification of viral RNA (RT-PCR). Cell culture and animal models are not available.

EM and IEM

The sensitivity of EM is limited to approximately 10^6 virus particles per g of stool, and HuCVs usually are excreted at lower concentrations than this during illness. EM detects only a minority of HuCV infections. IEM may improve the sensitivity of EM by 10 to 100 times. The prototype Norwalk virus was discovered using IEM (Kapikian et al., 1972).

Immunoassays

Antigen detection ELISAs based on hyperimmune sera or MAbs to virus-like particles or bacterially expressed capsid proteins of HuCVs have been developed and are commercially available for norovirus detection (Burton-MacLeod et al., 2004; de Bruin et al., 2006; Jiang et al., 2000). The sensitivity and specificity of these ELISAs are still inferior to RT-PCR techniques. One major challenge for the development of a broadly reactive norovirus detection assay is the wide antigenic variation of noroviruses. Approaches to overcome this challenge include the identification of common antigenic epitopes on the norovirus capsid (Parker et al., 2005) and cross-immunization of animals with capsid proteins representing different (if possible, all) antigenic types (Jiang et al., 2000).

Antibody detection ELISAs to measure HuCV-specific antibodies (IgA, IgG, and IgM) in patient sera are used in serosurveys of HuCV infection (Farkas et al., 2006; Jiang et al., 2000). When acute- and convalescent-phase blood samples have been collected, detection of seroresponses, usually measured as a ≥4-fold increase of antibody titer, can be used to determine infection status (Farkas et al., 2003; Jiang et al., 2000).

RT-PCR

RT-PCR is the most widely used assay for clinical and epidemiological studies and is currently the most sensitive assay for HuCV detection. The tremendous genetic diversity of HuCVs is a challenge for primer design. Primers targeting highly conserved regions of the genome, degenerated primers, and genogroup-specific primers are used. Highly conserved regions have been identified in ORF1 (RNA polymerase region) (Ando et al., 1995; Fankhauser et al., 2002; Farkas et al., 2004; Jiang et al., 1999c; Vinje and Koopmans, 1996), ORF2 (capsid) (Kojima et al., 2002; Noel et al., 1997a; Vinje et al., 2004), and the ORF1-ORF2 junction (Kageyama et al., 2003). Sequencing of the RT-PCR products allows typing and tracing of individual strains. Since a hotspot of RNA recombination has been found in the ORF1-ORF2 junction, recombinant viruses with distinct genotypes between ORF1 or ORF2 exist (Hansman et al., 2005c; Jiang et al., 1999b; Reuter et al., 2006; Vidal et al., 2006). Several quantitative real-time RT-PCR assays have also been described for the detection of HuCVs (Chan et al., 2006; Kageyama et al., 2003; Richards et al., 2004). RT-PCR is also useful for detecting HuCVs in environmental specimens, including drinking water and contaminated food (Beller et al., 1997; Le Guyader et al., 2004; Schwab et al., 1998).

Control and Prevention

Since noroviruses are the causative agents of the great majority of epidemic gastroenteritis worldwide, a vaccine or antiviral therapy is greatly needed. Unfortunately, at present neither of them is available. The high genetic and antigenic

variability of noroviruses, the short-termed, homotypic immunity, and the lack of a tissue culture system or suitable animal model are the biggest challenges for an effective vaccine design. Several approaches for the development of a norovirus vaccine have been investigated, including viral and subviral particles expressed in baculovirus, bacteria, yeast, and transgenic plants (Ball et al., 1996a; Tacket et al., 2000; Xia et al., 2007). The recent identification of HBGAs as receptors for noroviruses and advances in the characterization of HBGA-norovirus interaction opened a new area for antinorovirus drug development (Feng and Jiang, 2007).

Preventive strategies of calicivirus gastroenteritis focus on the elimination of the source of infection and interruption of transmission and include increased hygiene, disinfection of contaminated surfaces, identification of contaminated food or water, and removal of infected food handlers.

ASTROVIRUSES

Biology

The first astroviruses were identified in diarrhea stool specimens from children in 1975 by EM (Appleton and Higgins, 1975; Madeley and Cosgrove, 1975). Later astroviruses have been isolated from numerous animal species and recently grouped into two genera, *Mamastrovirus* and *Avastrovirus*, within the *Astroviridae* family. Astroviruses of mammals are usually associated with gastrointestinal illness, while avian astroviruses cause both intestinal and extraintestinal diseases, such as nephritis (Imada et al., 2000), hepatitis (Gough et al., 1984), and immunodeficiency (Qureshi et al., 2001).

Astroviruses are nonenveloped, 27- to 30-nm-diameter viruses with a characteristic star-like (astron = star in Greek) morphology. The viral genome is a positive-sense, single-stranded RNA of ~6.8 kb that contains three ORFs. ORF1a and ORF1b encode the nonstructural proteins, while ORF2 encodes the capsid precursor protein (Gibson et al., 1998; Jiang et al., 1993a; Lewis et al., 1994; Willcocks and Carter, 1993).

Eight human astrovirus (HAstV) antigenic types have been identified by immunofluorescence, neutralization, IEM, or genotyping (Belliot et al., 1997a; Herrmann et al., 1988; Hudson et al., 1989; Koopmans et al., 1998; Kurtz and Lee, 1984; Lee and Kurtz, 1994; Noel et al., 1995). HAstV-1 is the most frequently detected worldwide; however, multiple astrovirus serotypes cocirculate in the community (Guerrero et al., 1998; Noel et al., 1995; Schnagl et al., 2002).

Clinical Manifestation

The incubation period is 1 to 3 days after exposure, and astrovirus illness lasts approximately 4 to 5 days. Viral shedding may continue for several weeks (Mitchell et al., 1993). Clinical symptoms include diarrhea, vomiting, fever, malaise, and mild dehydration (Cruz et al., 1992; Guerrero et al., 1998). In temperate countries, astrovirus gastroenteritis peaks in the winter (Guix et al., 2002; Pang and Vesikari, 1999; Pennap et al., 2002; Putzker et al., 2000).

Pathogenesis

Astroviruses have been detected in villous epithelial cells and in macrophages of the lamina propria in small intestinal biopsy specimens from some children with chronic diarrhea (Phillips, 1988). Histopathology of small intestinal biopsy specimens from a 4-year-old bone marrow transplant recipient with prolonged astrovirus diarrhea demonstrated villous blunting, irregularity of surface epithelial cells, and inflammatory cell infiltration in the lamina propria. The highest concentration of astrovirus antigen was detected in enterocytes of the villus tips in duodenal and jejunal biopsy specimens (Sebire et al., 2004). Similarly, in animals, astrovirus infected mature villus epithelial cells and subepithelial macrophages in the small intestine, causing vacuolation of epithelial cells, followed by degeneration and cell death, leading to villous atrophy and crypt hyperplasia (Behling-Kelly et al., 2002; Gray et al., 1980; Kurtz, 1988; Snodgrass et al., 1979; Thouvenelle et al., 1995; Woode et al., 1984).

The immune response following astrovirus infection remains unclear. Most symptomatic astrovirus infections occur in children under 12 months of age, suggesting that maternal antibodies may not provide significant protection. The decreased infection rates after the first year of life could be due to an acquired immunity derived from previous infections, which tends to be mainly homotypic (Naficy et al., 2000).

Epidemiology

Human astrovirus infections occur worldwide and are primarily associated with pediatric disease. The incidence of astrovirus diarrhea ranges from 2% of children seeking medical care in Baltimore up to 15% of children with persistent (>14 days) diarrhea in Bangladesh (Bhattacharya et al., 2006; Fodha et al., 2006; Gaggero et al., 1998; Kotloff et al., 1992; Unicomb et al., 1998). Infection occurs mainly in children younger than 2 years of age and is frequently asymptomatic (Liu et al., 2004; Mitchell et al., 1993; Naficy et al., 2000). Seroprevalence studies show that 60 to 90% of school-aged children possess antibodies to astroviruses (Kobayashi et al., 1999; Koopmans et al., 1998; Kriston et al., 1996; Mitchell et al., 1999).

Diarrhea outbreaks among children associated with astrovirus have been reported in communities, child care centers (Mitchell et al., 1995; Mitchell et al., 1993), schools (Oishi et al., 1994), and hospitals (Ashley et al., 1978; Kurtz et al., 1977). Astroviruses may account for 0.8 to 7% of hospitalizations of children for diarrhea in both developed and developing countries (Colomba et al., 2006; Ellis et al., 1984; Marie-Cardine et al., 2002; Palombo and Bishop, 1996; Pazdiora et al., 2006; Shetty et al., 1995). Astroviruses have also been reported to be important causes of diarrhea among the immunocompromised (Cox et al., 1994; Cubitt et al., 1999; Grohmann et al., 1993; Noel and Cubitt, 1994; Wood et al., 1988).

Outbreaks of astrovirus gastroenteritis in adults have been reported in elder care facilities (Gray et al., 1987; Lewis et al., 1989; Marshall et al., 2007; Midthun et al., 1993) and among military recruits (Belliot et al., 1997b). A large outbreak of food-borne astrovirus infection in Japan that was linked to contaminated food from a common supplier involved thousands of children and adults (Oishi et al., 1994). In a recent study, 0.5% of 941 gastroenteritis outbreaks of suspected viral etiology that were studied between 1994 and 2005 in The Netherlands were linked to astroviruses (Svraka et al., 2007).

Diagnosis

The method used for the detection of astroviruses significantly affects the estimation of astrovirus-related diarrhea. Initial studies of the incidence of astrovirus infection were based on virus detection by EM. Curently available detection assays rely upon ELISA and RT-PCR.

Cell Culture

In the presence of trypsin in the culture medium, astroviruses can be propagated in various cell lines (Lee and Kurtz, 1981; Taylor et al., 1997a), of which Caco-2, T84, and PLC/PRF/5 are the most effective for virus isolation from clinical

samples (Brinker et al., 2000). Cell culture also has been utilized to amplify astroviruses from clinical or environmental samples before detection by other methods (Chapron et al., 2000; Mustafa et al., 1998; Pinto et al., 1994).

EM and IEM

The sensitivity of EM is less than that of immunoassays or RT-PCR. In negatively stained EM preparations, only ~10% of the virions have the typical star-like morphology, which requires samples with high virus load for confident diagnosis (Madeley, 1979). IEM enhances astrovirus detection but may require homotypic antisera for optimal application.

Immunoassays

ELISAs with group-specific monoclonal antibodies and group-common polyclonal antibodies have been developed for antigen detection of astroviruses (Herrmann et al., 1988; Herrmann et al., 1990; Moe et al., 1991). By utilizing type-specific hyperimmune antisera as the capture antibody and the group-specific MAb as the detector antibody, a typing ELISA has been described (Noel et al., 1995). These antigen detection ELISAs facilitated epidemiologic studies, demonstrating that astroviruses are a significant cause of diarrhea in children worldwide (Glass et al., 1996b). Today, several commercial astrovirus antigen detection kits are available (Dennehy et al., 2001; McIver et al., 2000; Putzker et al., 2000), but they might be less sensitive than RT-PCR and the true prevalence of astroviruses could be underestimated if only immunoassays are used for detection (Dalton et al., 2002).

Serology

A virus neutralization test has been developed and used to confirm serotype designation of astroviruses and detect serotype-specific antibodies in sera (Koopmans et al., 1998). Type-specific recombinant capsid proteins of HAstV-1, HAstV-3, and HAstV-6, produced in a baculovirus system, have been utilized to develop microimmunofluorescence assay or ELISA to detect antiastrovirus antibodies in human sera (Kriston et al., 1996; Mitchell et al., 1999). The development of comparable assays for all astrovirus serotypes will be useful for further definition of the epidemiology of astroviruses.

RT-PCR

Primers targeting both type-common and type-specific regions of astroviruses have been utilized for RT-PCR assays (Belliot et al., 1997a; Grimm et al., 2004; Jonassen et al., 1995; Matsui et al., 1998; Mitchell et al., 1995; Noel et al., 1995; Saito et al., 1995). Recently, nucleic acid sequence-based amplification and real time RT-PCR assays for the detection of astroviruses in clinical samples also have been described (Royuela et al., 2006; Zhang et al., 2006). Sequencing of the RT-PCR products and phylogenetic analysis is often used for further strain classification; however, the outcome of this analysis can vary based on the choice of the genomic region. Analysis of ORF1a sequences of astrovirus serotypes 1 to 7 indicated two distinct genogroups, with genogroup A consisting of serotypes 1 to 5 and genogroup B including serotypes 6 and 7. In contrast, when analyzing nucleotide sequences from ORF1b or ORF2, serotypes cluster nearly equidistant (Belliot et al., 1997a). Recombination events may be responsible for this difference in genetic relationship (Walter et al., 2001).

Control and Prevention

There is no antiviral available for astroviruses. There has been no development of an astrovirus vaccine. Since the majority of cases of severe astrovirus diarrhea occur in very young children (<6 months old), an astrovirus vaccine targeted to this age group is necessary. Until an effective vaccine and/or antiviral is developed, interruption of transmission by improved personal hygiene and environment disinfection should be the major focus of prevention.

ENTERIC ADENOVIRUSES

Biology

Human adenoviruses have been linked with a number of diseases, including respiratory illness, conjunctivitis, and diarrhea. They are classified into the *Adenoviridae* family, *Mastadenovirus* genus. Based on differences in viral hemagglutination, oncogenic properties, and the size, composition, homology, and organization of their genomes, human adenoviruses are grouped into 6 subgroups (A to F) and further differentiated into at least 51 human adenovirus serotypes (De Jong et al., 1999). Many adenoviruses are readily isolated from human stools, but their role in acute gastroenteritis is unclear. Two serotypes from subgroup F, Ad40 and Ad41, however, have been clearly associated with infantile diarrhea (de Jong et al., 1983; Denno et al., 2005; Gary et al., 1979; Nguyen et al., 2007; Uhnoo et al., 1983; Uhnoo et al., 1984; Yolken et al., 1982) and are referred to as enteric adenoviruses (EAs). Adenoviruses are nonenveloped viruses that are 70 to 100 nm in diameter. Mature virions consist of a DNA-containing core surrounded by a protein shell (capsid). The genome is a linear, double-stranded DNA. The adenovirus 40 (Ad40) genome is 34,214 bp long (Davison et al., 1993). There are 11 structural proteins, three of which are coat proteins (hexon, penton, and fiber) that have important clinical significance. These proteins are involved in viral entry (Bergelson et al., 1997; Louis et al., 1994) and intracellular transportation and contain the major neutralization epitopes (Roy et al., 2005; Watson et al., 1988).

Pathogenesis

There is limited information on the pathophysiologic mechanism involved in EA infections. Observations from a human case suggest that the nuclei of villous epithelial cells are the sites of virus concentration and that the microvillus structure is altered (Phillipps, 1988). The incubation period is 3 to 10 days. EA infections are less likely to be associated with high fever or dehydration but more likely to cause prolonged illness than rotavirus infection (Grimwood et al., 1995; Uhnoo et al., 1984). Diarrhea lasts from 6 to 9 days in most cases, with a range from 4 to 23 days (Kotloff et al., 1989; Lin et al., 2000; Uhnoo et al., 1986; Van et al., 1992). Vomiting and fever may precede or accompany the diarrhea. EAs have rarely been associated with fatal disease (Whitelaw et al., 1977). An association between EAs and celiac disease has been postulated but not proven (Kagnoff et al., 1987; Lahdeaho et al., 1993).

Epidemiology

The incidence of EA-related gastroenteritis differs considerably in various studies and locations. EAs cause 2 to 22% of diarrhea episodes, mainly in children younger than 4 years of age, but are also frequently detected in stool specimens of children with asymptomatic infection (Basu et al., 2003; Caeiro et al., 1999; Cunliffe et al., 2002; Fukuda et al., 2006; Giordano et al., 2001; Grimwood et al., 1995; Mickan and Kok, 1994; Nguyen et al., 2007; Noel et al., 1994; Phan et al., 2005a; Steele et al., 1998; Uhnoo et al., 1990b; Vizzi et al., 1996; Wang and Chen, 1997). Antibody prevalence to EAs

has been shown to increase from 20% during the first 6 months of life to ≥50% by the third to fourth year of life (Shinozaki et al., 1987). Both EA types appear to be widespread and to cause endemic diarrhea. Outbreaks have been reported in orphanages, hospitals, and child care settings (Chiba et al., 1983; Paerregaard et al., 1990; Richmond et al., 1979; Taylor et al., 1997b; Van et al., 1992). Seasonal and year to year shifts from one serotype to another have been shown at several sites (Grimwood et al., 1995; Mickan and Kok, 1994). The seasonality of EA infections is uncertain.

Diagnosis

EAs were also called "fastidious" adenoviruses due to the inability to cultivate them in conventional cell cultures. Later, they were successfully propagated in HEK 293 cells, an adenovirus type 5-transformed cell line (Takiff et al., 1981), or in Caco-2 cells (Pinto et al., 1994). Diagnostic assays based on reagents derived from these cell culture-adapted viruses are now available. Diagnosis of EA infection can be done by EM, latex agglutination (Grandien et al., 1987), ELISA (Herrmann et al., 1987; Vizzi et al., 1996; Wood et al., 1989), and PCR (Allard et al., 1990; Avellon et al., 2001; Rousell et al., 1993; Xu et al., 2000). Some of these tests are commercially available. Restriction enzyme analysis of adenovirus DNA extracted from virus isolates has been used for classifying individual isolates (Takiff et al., 1981; Uhnoo et al., 1984), but it has been replaced with more convenient genotyping methods (Kidd et al., 1996; Li et al., 1999; Soares et al., 2004).

OTHER ENTEROPATHOGENIC VIRUSES

A number of different viruses other than those discussed above have been identified in stool samples from humans with acute gastroenteritis, but their role and significance in causing illness are still not well established. With the exception of the Aichi virus, these viruses cannot be grown in tissue culture.

Coronaviruses and Toroviruses

Coronavirus and *Torovirus* are the two genera of the *Coronaviridae* family of enveloped, single-stranded, positive-sense RNA (25 to 31 kb) viruses. Coronaviruses infect the respiratory and gastrointestinal tracts and are a recognized cause of common colds in humans. A more serious, often fatal respiratory illness is caused by the severe acute respiratory syndrome coronavirus that had one major epidemic in 2002 to 2003. Diarrhea has been described as a common symptom of severe acute respiratory syndrome, presenting most frequently in the first week of illness (Leung et al., 2003). Although coronaviruses such as transmissible gastroenteritis virus of pigs and canine coronavirus are well-established gastrointestinal pathogens of animals, coronaviruses have only sporadically been associated with cases of human gastrointestinal illness, particularly in newborns (Clarke et al., 1979; Gerna et al., 1985; Zhang et al., 1994).

The first torovirus described was the Breda virus in cattle (Woode et al., 1982), followed by the Berne virus in horses (Weiss et al., 1983). Data obtained in virus neutralization tests with the Berne virus indicated that toroviruses are present in several other animal species (Weiss et al., 1984). Torovirus-like particles were first described in human stools collected from children and adults with diarrhea in 1984 using EM (Beards et al., 1984). The role of bovine toroviruses in causing diarrhea in cattle has been established by experimental inoculation of gnotobiotic calves (Pohlenz et al., 1984) and prevalence studies (Duckmanton et al., 1998; Ito et al., 2007; Koopmans et al., 1991). Inoculation of foals with the Berne virus did not result in clinical symptoms; however, the animals developed neutralizing antibodies (Weiss et al., 1984). Similarly, in piglets, only virus shedding can be detected without firm clinical symptoms (Kroneman et al., 1998). Despite several studies that indicated the role of toroviruses in human gastroenteritis (Jamieson et al., 1998; Koopmans et al., 1993; Koopmans et al., 1997; Krishnan and Naik, 1997; Lodha et al., 2005; Waters et al., 2000), their significance is not well established. Since human toroviruses have not been isolated in cell culture and reference antisera are not routinely available, diagnosis is limited to EM examination of stool specimens. Due to the pleomorphic nature of torovirus particles, the EM results must be interpreted with caution, because pieces of sloughed intestinal epithelium may appear as torovirus-like particles. A confirmation of EM results by IEM, ELISA (Koopmans et al., 1993), or RT-PCR (Duckmanton et al., 1997) is necessary.

Picobirnaviruses

Picobirnaviruses belong to the *Picobirnavirus* genus within the *Birnaviridae* family, which contains double-stranded RNA viruses with two genomic segments. Picobirnaviruses are smaller in size and have shorter genomic segments than those of other members of the family. Picobirnaviruses were first described in 1988 in stool samples of humans and rats (Pereira et al., 1988a; Pereira et al., 1988b) by polyacrylamide gel electrophoresis (PAGE), and since then, they have been detected in fecal samples of many domestic and wild animals (Buzinaro et al., 2003; Gallimore et al., 1993; Haga et al., 1999; Leite et al., 1990; Ludert et al., 1991; Masachessi et al., 2007; Pongsuwanna et al., 1996). Picobirnaviruses are nonenveloped, 35- to 40-nm-diameter, round particles with a smooth surface. The sizes of the two genomic segments vary from 2.3 to 2.6 kb for genomic segment 1 and 1.5 to 1.9 kb for genomic segment 2 among different strains. Segment 2 encodes the RNA-dependent RNA polymerase, and segment 1 possibly encodes the viral capsid protein. Based on segment 2 sequences, human picobirnaviruses have been divided into two major genogroups (Rosen et al., 2000). Since the size of picobirnavirus genomic segments is in the range of the size of rotavirus genomic segments, picobirnaviruses are often "accidentally" detected during rotavirus surveillance by PAGE. Other than PAGE, RT-PCR is the most widely used method for picobirnavirus detection (Rosen et al., 2000). Since picobirnaviruses have been detected in the feces of humans or animals with and without diarrhea (Cascio et al., 1996; Gallimore et al., 1995; Giordano et al., 1999; Gonzalez et al., 1998; Ludert and Liprandi, 1993) and in many cases diarrhea stool samples were also positive for noroviruses or rotaviruses (Banyai et al., 2003; Bhattacharya et al., 2007), their role in diarrheal disease is not clear.

Aichi Virus

Aichi virus was first detected in stool samples of patients during an investigation of oyster-associated gastroenteritis outbreaks in 1989 in Japan (Yamashita et al., 1991). Since then, Aichi virus has been detected in stools of gastroenteritis patients many times in Asia, Europe, and South America (Oh et al., 2006; Yamashita et al., 2001; Yamashita et al., 1993; Yamashita et al., 1995; Yamashita et al., 2000), but the significance of Aichi virus as a cause of gastroenteritis in humans remains unknown.

Aichi virus represents a new genus, *Kobuvirus*, in the *Picornaviridae* family (Van Regenmortel, 1999). Based on

phylogenetic analysis of a 519-bp genome segment in the 3C-3D junction region, Aichi virus isolates were further classified into genotypes A and B (Yamashita et al., 2000). Aichi virus grows well in monkey kidney cells (Vero, BS-C-1) and causes a typical cytopathic effect. A new species of the genus, the bovine kobuvirus, has been isolated in 2003 from HeLa cell cultures that possibly had been contaminated with this virus from the calf serum used to maintain the cells (Yamashita et al., 2003). The Aichi virus particles are nonenveloped, ~30 nm in diameter, encapsidating a positive-sense, single-stranded RNA genome of ~8.2 kb, with a poly(A) tail. The genome, with an organization typical of picornaviruses, contains a single large ORF that encodes a polyprotein that is processed by protease cleavage to individual structural and nonstructural proteins (Yamashita et al., 1998). Of the three structural proteins (VP0, VP1, and VP3), VP0, a 42-kDa protein, strongly reacts with convalescent-phase patient sera. For diagnosis, RT-PCR (Yamashita et al., 2000), antigen detection ELISA (Yamashita et al., 1993), and antibody detection ELISA (Yamashita et al., 2001) have been described.

Others

Some diarrhea outbreaks and sporadic cases have also been reported to be associated with enteroviruses (Abe et al., 2000; Joki-Korpela and Hyypia, 1998; Patel et al., 1985; Phan et al., 2005b; Townsend et al., 1982) that belong to the *Picornaviridae* family and infect the host via the gastrointestinal tract and are excreted in stool for several weeks. These viruses can also spread from the primary infection site of the intestines via the bloodstream to several organs and may cause meningitis, paralytic poliomyelitis, myocarditis, pericarditis, hemorrhagic conjunctivitis, and pleurodynia.

Human immunodeficiency virus infection of the gut-associated lymphoid tissue and enterocytes can result in chronic diarrhea in many AIDS patients (Nelson et al., 1988; Rabeneck, 1994), often complicated by chronic infection with other enteric pathogens (Wallace and Brann, 2000). In the immunocompromised host, cytomegalovirus and herpes simplex virus infection often result in colitis and esophagitis (Carter et al., 2006; Kandiel and Lashner, 2006).

CONCLUSION

Viral gastroenteritis remains an important infectious disease worldwide. Most of the gastroenteritis viruses cause sporadic diseases in children, with rotaviruses being the most important cause of severe diarrhea, which accounts for a significant number of fatal infections in children, mainly in resource-poor countries. Some of the gastroenteritis viruses, such as noroviruses, sapoviruses, and astroviruses, are also common causes of outbreaks of acute gastroenteritis in children, with noroviruses being the most common cause of large outbreaks of acute gastroenteritis in all age groups. Viruses causing gastroenteritis are mainly transmitted through the fecal-oral route by person-to-person contact, through contaminated environmental surfaces, and/or by contaminated water or food sources. Crowdedness is a significant risk factor, and viral gastroenteritis outbreaks most commonly occur in such settings as child-care centers, nursing homes for the elderly, cruise ship lines, and the military. Due to the wide genetic and antigenic variation and the lack of optimal cell culture or animal model for most of the gastroenteritis viruses, their study remains difficult. With the introduction of molecular cloning techniques and the development of new diagnostic methods, our understanding of the role of these agents in human diseases has been significantly improved in the past decade, including the recent discovery of the viral receptors for human noroviruses and linking HBGAs and sensitivity of the host to norovirus infection. With continuing research, new discoveries and their application in disease control and prevention are expected to be forthcoming in the near future. The worldwide increase in awareness and monitoring of acute gastrointestinal illness also will help to further define the role of coronaviruses, toroviruses, picobirnaviruses, and Aichi virus and possibly link other viral agents to gastroenteritis in humans. The recent introduction of rotavirus vaccines to the international markets is a promising start and hopefully will be followed by similar strategies for other viral causes of acute gastroenteritis.

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Waterborne Hepatitis

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20

Viral hepatitis is the general term for inflammatory disease of the liver caused by at least five different viruses, with hepatitis A, B, C, D, and E viruses having a definite association with acute viral hepatitis. The waterborne hepatitis viruses, hepatitis A virus (HAV) and hepatitis E virus (HEV), both cause acute and generally self-limiting infections, and although fulminant hepatitis can occur, patients do not progress to long-term carrier status. At the time of clinical presentation, patients may be excreting large amounts of infectious virus, and HAV in particular is very easily transmitted from person to person and through large-scale, common-source outbreaks which are often traced to contaminated food. This contrasts with the blood-borne hepatitis viruses which progress to chronic infections in many cases, with serious long-term sequelae, but with a lower chance of person-to-person transmission other than through parenteral contact and, in the case of hepatitis B virus (HBV) and hepatitis D virus, sexual contact. As such, diagnosis of HAV or HEV is important for appropriate clinical management of patients, including exclusion of the potentially more serious HBV, hepatitis C virus (HCV), and hepatitis D virus, and in detection of outbreaks, HAV in developed countries, and both HAV and HEV in developing countries.

Despite great differences among the viruses, the clinical presentation of viral hepatitis is quite uniform in the acute phase, and differential diagnosis is therefore dependent on specific tests for each of the viruses. The selection of appropriate diagnostic tests to be used in the first instance should be based on an assessment of the most likely risk factors for each infection, but interpretation of test results must also take into account the predictive value of the tests performed, which may vary widely.

This chapter will address the biology and epidemiology of the waterborne viruses, HAV and HEV, the value of diagnostic assays for each virus in different settings, and the current and future prospects for prevention and control of HAV and HEV infections.

BIOLOGY OF HAV AND HEV

Both HAV and HEV are small viruses with genomes of single-stranded, positive-sense RNA (Fig. 1), with their icosahedral viral particles lacking a lipid envelope. HAV was first propagated in cell culture almost 30 years ago (Provost and Hilleman, 1979), and extensive studies (including the use of infectious molecular clones) have revealed many details of its replication, protein processing, and assembly. This also has been aided by inferences from the study of related members of the picornavirus family, such as poliovirus. In contrast, the infectivity of molecular clones of HEV was demonstrated first in primates (Emerson et al., 2001), and only very recently has replication in cell culture been convincingly demonstrated (Emerson et al., 2004). The low level of in vitro replication obtained for HEV hampers detailed studies, and apart from an avian genotype of HEV, there are no closely related viruses from which we can infer other details of the replication cycle.

Biology of HAV

HAV is the type member of the *Hepatovirus* genus in the family *Picornaviridae*. Replication of HAV begins with calcium-dependent attachment to one or more specific cellular receptors, of which the mucin-like molecule HAVcr1/TIM1 appears to be of key importance (Kaplan et al., 1996; Feigelstock et al., 1998; Tami et al., 2007). Internalization occurs rapidly, but uncoating (leading to release of the viral RNA) is very protracted, taking up to 12 h (Wheeler et al., 1986; Bishop and Anderson, 2000). The positive-strand RNA genome encodes only a single open reading frame (ORF), which is translated to yield a giant polyprotein, and the virus-encoded 3C protease cleaves the polyprotein at a number of sites, liberating 8 proteins in the first instance, including the RNA-dependent RNA polymerase (RDRP, or $3D^{pol}$) and protease, $3C^{pro}$. The small VPg protein (part of 3AB) then serves as a primer for the synthesis by RDRP of a complementary, negative-strand RNA and, subsequently, for further copies of positive-strand RNA. These identical "daughter" positive-strand RNAs serve as templates for further transcription to amplify the RNA pool, as messenger RNA for synthesis of viral proteins, and finally, as new genomes for encapsidation within the viral particle (see Rueckert, 1990, for a comprehensive review of picornaviral replication). In common with other RNA viruses, HAV replication induces production of interferon (IFN), but recent studies have shown that the 3ABC protease precursor is targeted to mitochondria where it cleaves an essential component of the IFN signaling pathway, thereby ablating the type I IFN response (Yang et al., 2007).

The HAV particle is initially composed of three proteins, VP0, VP1-2A (also known as PX), and VP3 (Anderson and

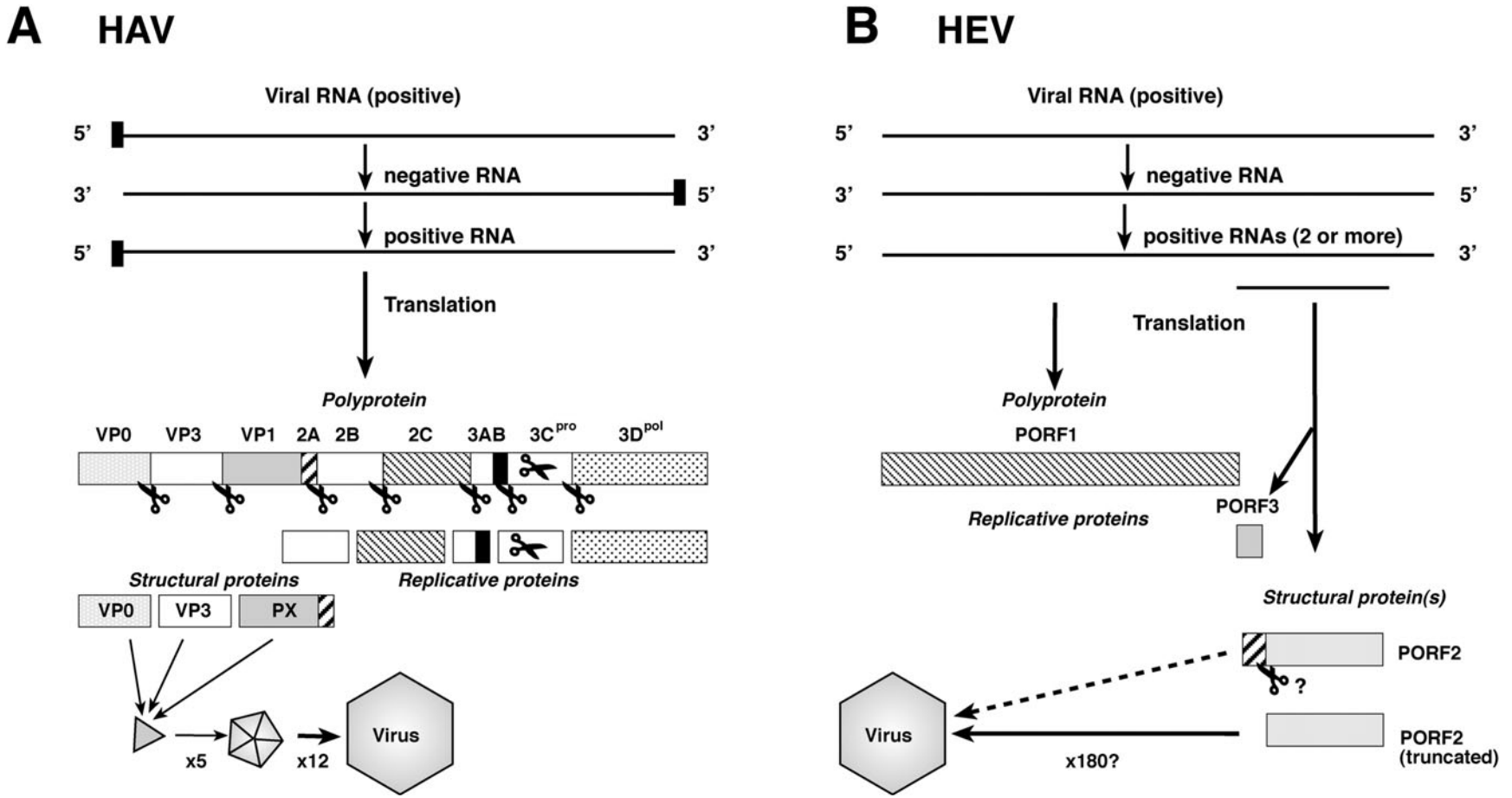

FIGURE 1 Genome replication and the encoded proteins of HAV (A) and HEV (B). Both viruses have positive-strand RNA genomes of around 7,500 nucleotides. HAV replication proceeds via transcription from the genome to give full-length negative- and then positive-strand RNA, which can either be assembled into the virus particle or used to translate further copies of a single, giant polyprotein which is processed by viral protease to yield the replicative proteins and capsid proteins. Assembly of five copies of each of the three capsid proteins (VP0, VP3, and PX) into pentamers and then 12 pentamers into capsids is required to form the antigenic sites of the virus. (B) Details of HEV replication are unknown, but it most likely produces a full-length negative-strand RNA and then full-length positive-strand RNA (new viral genomes and mRNA for the ORF1 polyprotein) as well as a single subgenomic messenger RNA which is used to translate the ORF2 (capsid) and ORF3 proteins. Processing of the ORF1 polyprotein yields the replicative proteins, but the precise locations of cleavage sites is unknown. Cleavage of full-length PORF2 results in assembly of VLPs from the truncated product, but it is not known whether the truncated or full-length PORF2 is normally involved in virus assembly. PORF3 is dispensable for replication in vitro but not in vivo.

Ross, 1990), which are liberated from the polyprotein by 3C (Martin et al., 1995) (Fig. 1A). Five copies of each of the three proteins associate to form pentamers, and 12 copies of the pentamer form virions (with viral RNA) or empty capsids with the same antigenicity. Following assembly, 2A is removed from VP1 by cellular enzymes and/or 3C protease, and in the final maturation step, VP0 is cleaved via an RNA-dependent mechanism to yield VP2 and VP4 (Bishop and Anderson, 1993); this maturation cleavage is not necessary for infectivity but affects the rate of virus uncoating, with particles having cleaved VP0 being uncoated more rapidly (Bishop and Anderson, 2000). Virus is released from cells without cell lysis and is at least partly sensitive to brefeldin A, implicating cellular vesicular transport pathways (Blank et al., 2000).

Large quantities of HAV are found in bile, which is likely to provide the majority of virus in feces (Schulman et al., 1976; Krawczynski et al., 1981). Interestingly, while HAV is preferentially released at the apical surface in a model of polarized enteric epithelial cells (Caco2), consistent with the direct release of virus into the bile canaliculi and, hence, the gut (Blank et al., 2000), very recent studies have shown that it is preferentially released at the basolateral pole in a model of polarized hepatocytes, which would instead release virus into the bloodstream (Snooks et al., 2008), suggesting an indirect route for excretion into bile and feces.

Diagnosis and immunization for hepatitis A are not complicated by strain differences, despite significant levels of amino acid variation in the capsid proteins, because HAV exists as a single serotype worldwide. This is especially notable in the case of the highly divergent AGM-27, which is considered a true simian strain of HAV, causing disease in African green monkeys and other lower primates (Emerson et al., 1991; Tsarev et al., 1991). The conservation of antigenic sites but low pathogenicity in chimpanzees for this strain suggest that it could have potential as a live, naturally attenuated vaccine (Emerson et al., 1996). The antigenic sites of HAV are formed through the complex interactions of the proteins within and between pentamers (Stapleton et al., 1993), which has hampered the production of HAV antigenic material through recombinant DNA techniques.

Biology of HEV

HEV is the type species of the new genus *Hepevirus*, family *Hepeviridae* (Emerson et al., 2005a). A related virus of chickens (avian HEV) (Haqshenas et al., 2002; Huang et al., 2002) is the only other family member known at this time, and is currently classified within the same genus. While our knowledge of HEV replication is currently inadequate, the mechanism of RNA replication and transcription and the assembly pathway are certainly very different from those of HAV (Fig. 1B).

The HEV genome contains three ORFs, organized as 5′-ORF1-ORF3-ORF2-3′ (Fig. 1B), with ORF3 and ORF2 largely overlapping. The development of functional "replicons" of HEV RNA recently has allowed the identification of a single subgenomic RNA that functions as a bicistronic mRNA for translation of both ORF2 and ORF3 proteins (Graff et al., 2006), but the mechanism for transcription of this subgenomic RNA is not yet known.

Translation of ORF1 yields a polyprotein (PORF1) containing sequence motifs consistent with RDRP, RNA helicase, methyltransferase, and protease activities (Tam et al., 1991), although none of these activities has been directly demonstrated and the sizes of mature proteins are unknown. The proteins within PORF1 are sufficient for genome replication.

ORF2 encodes the capsid protein, PORF2, and expression of truncated forms of this protein (lacking the first 111 amino acids) in insect cells leads to the assembly of virus-like particles (VLPs, or similar particles described as subviral particles) (He et al., 1993; Tsarev et al., 1993; Li et al., 1997b). It is not known whether the authentic virus particle contains a truncated PORF2 (as in VLPs) or rather the full-length PORF2 in association with other factors, such as the viral RNA, which may promote its proper assembly into viral particles. Notwithstanding this, VLPs produced using recombinant DNA techniques appear to possess the important antigenic epitopes of the virus (Li et al., 1997b; Zhang et al., 1997; Robinson et al., 1998), and the three-dimensional structure of these VLPs has yielded important clues regarding the structure and assembly of HEV (Xing et al., 1999; Li et al., 2005).

ORF3 encodes the small, highly basic PORF3 protein, which has been reported to associate with both PORF2 (Jameel et al., 1996) and the cytoskeleton (Zafrullah et al., 1997) with multiple effects on the host cell. PORF3 is not required for efficient replication in cell culture (Emerson et al., 2004), but a *cis*-reactive RNA structural element (*cre*) within ORF3 is required (Graff et al., 2005). However, PORF3 is essential for infectivity in macaques (Graff et al., 2005), which indicates that it is likely to play a major role in virus-host interactions. The HEV PORF3 is also immunogenic, with many patients producing a strong but transient antibody response against the protein.

It has been assumed that, following assembly, HEV particles would be released from the hepatocyte via the apical surface to reach the bile and gut for transmission (Anderson and Cheng, 2005); however, recent results suggest that this is not the case for HAV (Snooks et al., 2008) and it is not known whether HEV may follow a similar, indirect pathway as for HAV.

Genetic and antigenic variation between HEV strains is far more pronounced than for HAV. Early studies identified the prototype Burmese (Reyes et al., 1990) and Mexican (Huang et al., 1992) strains and showed that a number of epitopes were type specific (Yarbough et al., 1991), but more recent studies have greatly expanded the known diversity of HEV. This list now includes putative "genotypes" (as defined by Wang et al., 1999, on the basis of relatively conserved ORF1 sequences) representing the Burmese and related strains (including African, Eastern European, and most Chinese strains) as genotype 1, the Mexican strain as genotype 2, the swine HEV strain (Meng et al., 1997) and closely related strains isolated from patients infected in the United States (Kwo et al., 1997; Schlauder et al., 1998) as genotype 3, and distinct isolates from China (Wang et al., 1999) and Taiwan (Hsieh et al., 1998) as genotype 4. Avian HEV (Haqshenas et al., 2002; Huang et al., 2002), the causative agent of big liver and spleen disease in chickens, may be considered genotype 5 but is much more divergent than seen for the mammalian HEV genotypes. As ORF1 encodes replicative proteins, it is not surprising that even greater variation is seen in the ORF2 and ORF3 proteins, with ORF3 varying by more than 50% between many strains of mammalian HEV. This genetic and antigenic diversity has important implications for epidemiology, diagnosis, and control that will be discussed in detail below.

PATHOGENESIS

Clinical Characteristics

Acute infections with any of the hepatitis viruses, including HAV and HEV, cannot be distinguished on clinical characteristics or pathological examinations. However, HEV

infection is unique among the hepatitis viruses in being associated with a high mortality during pregnancy due to fulminant hepatitis with a very rapid onset, approaching 30% in the third trimester, and the rate of fulminant hepatitis for HEV outside of pregnancy is probably around 10-fold higher than for HAV (1% versus 0.1%) (Balayan, 1997). The reasons for such severe outcomes in HEV infection are not known, but it is clear that women should take all possible precautions to avoid exposure to HEV during pregnancy. A major risk factor in this regard is travel to areas of endemicity, such as India and Pakistan. In epidemic and emergency settings, such as refugee camps, the burden of fulminant HEV among pregnant women can be severe (Boccia et al., 2006; Guthmann et al., 2006).

Infection with HAV or HEV can result in a broad range of clinical outcomes, from subclinical infections (especially in children) through to fulminant hepatitis. However, clinical and even fulminant hepatitis A do occur in children, and there is no reason to exclude the enterically transmitted hepatitis viruses from diagnostic consideration on the basis of patient age.

Clinical presentation of acute viral hepatitis commonly begins with nonspecific, "flu-like" symptoms such as fever, headache, anorexia, nausea, and abdominal discomfort. The first distinctive sign of hepatitis is usually dark urine, followed by pale feces and jaundice (yellow discoloration of the skin and sclera); however, some patients will not show visible signs of jaundice despite severe symptoms. Physical examination will usually reveal an enlarged, tender liver (Anderson and Shrestha, 2009).

Liver function tests are an important adjunct to diagnosis, with raised levels of serum bilirubin, aspartate aminotransferase, and alanine aminotransferase being detected at the time of onset, usually resolving after a period of 3 to 4 weeks. Normalization of liver enzymes usually marks complete recovery; however, many patients will report an intolerance to fatty foods which may last for years. When acute hepatitis is strongly suspected on the basis of clinical symptoms combined with likely exposure history, it is appropriate to test for specific hepatitis virus infections without waiting for the results of liver function tests.

Relapses are rare for hepatitis A, occurring in around 7% of patients beyond 1 month (Sjogren et al., 1987), and prolonged disease with hepatitis E also has been observed in some areas of endemicity.

While both HAV and HEV have low rates of fulminant hepatitis, the onset of encephalopathy can be quite rapid (around 7 days from onset of dark urine for hepatitis A), with laboratory examination revealing an increase in prothrombin time and increasing bilirubin levels (Ross et al., 1991).

Natural History of Virus Infection

The generalized course of infection and serological responses for both HAV and HEV is shown in Fig. 2. Following ingestion of contaminated water or food, it is presumed that infection is initiated via cells lining the alimentary tract, but while some evidence of HAV replication in intestinal tissues has been reported (Asher et al., 1995), it is not clear whether this is essential. In addition, it has been shown that HAV is released almost exclusively via the apical domain from infected enterocytes (Blank et al., 2000), which would not seed virus directly into the bloodstream. As such, it is probable that virus enters the bloodstream via transcytosis across the enteric mucosa.

Virus then spreads to the liver, eventually infecting a large proportion of the hepatocyte population but without causing direct cytolytic damage. After an incubation period of 4 to 6 weeks after first exposure to the virus, liver damage results and is thought to be mediated by the cellular immune response to the viruses, with infections in young children generally following a benign course. Virus is excreted through bile to the gut (Schulman et al., 1976; Krawczynski et al., 1981), and very high titers of infectious HAV will be present in most patients at the onset of illness (Coulepis et al., 1980), while titers of HEV are lower. Virus is generally cleared within several weeks, although prolonged excretion of both viruses has been reported in some cases. In general, titers of excreted virus will be highest before the onset of obvious symptoms.

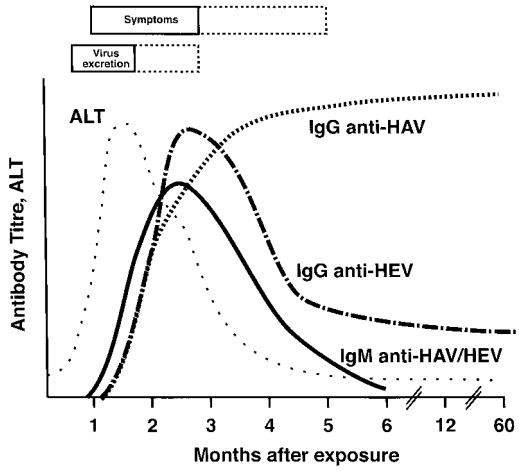

FIGURE 2 Serological and virological courses of infection with HAV or HEV. For HAV, the serological responses shown are typical of those detected with numerous commercially available assays. For HEV, the serological responses shown are those that probably occur in most patients, but the detection of these responses will vary widely depending on the assays used. High levels of HAV-specific IgG provide lifelong protection from reinfection, but HEV-specific IgG declines rapidly during the first 6 months and might not persist at protective levels for life. ALT, alanine aminotransferase.

Although the vast majority of progeny virus is excreted in the feces, both HAV and HEV produce a viremia that may last for some weeks around the time of clinical presentation. Transmission of HAV via blood products has been demonstrated (Mosley et al., 1994; Soucie et al., 1998), and inactivation procedures to eliminate HAV as well as the more obvious blood-borne viruses, such as HBV, HCV, and human immunodeficiency virus, are clearly required in the manufacture of blood products (Lemon, 1994, 1995). Fortunately, such processes have now been validated for HAV, and further transmission is unlikely (Lemon 1995; Biesert et al., 1996; Adcock et al., 1998).

Immunity

High titers of immunoglobulin G (IgG) and IgM (and IgA) antibodies are produced by the time of disease onset. For HAV, IgM will often be detectable prior to IgG and IgG will continue to rise for some weeks after onset, whereas HEV-specific IgM and IgG will both peak at around the time of onset. Although the viruses infect via mucosal surfaces, it is clear that circulating specific IgG is protective for both viruses, provided that there is a sufficient titer of the appropriate

antibody specificity. Infection with HAV produces lifelong immunity with the maintenance of high levels of specific IgG (Fig. 2), and studies with immune gamma globulin (IG) prophylaxis have allowed minimal protective levels of antibody to be determined (Stapleton et al., 1985). It is also clear from passive immunization studies with high-titer macaque IG that antibody is sufficient to confer protection against HEV (Tsarev et al., 1994). However, titers of IgG anti-HEV appear to decline by around 90% in the first 6 months after infection before stabilizing, and it is not yet known whether levels of anti-HEV remain high enough to confer protection for life. Indeed, IG prepared from humans has proven ineffective, presumably due to low titers of neutralizing antibody (Khuroo and Dar, 1992; Chauhan et al., 1998). Protective monoclonal antibodies have been isolated from an immune chimpanzee (Schofield et al., 2000), and this approach could potentially provide a source of antibodies for passive immunization, although cost is likely to be prohibitive for use in developing countries. The recent development of an enzyme-linked immunosorbent assay (ELISA) that may be specific for HEV neutralizing antibody (Zhou et al., 2004) is likely to be an important tool in the ongoing clinical development of HEV vaccines as well as in selection of appropriate IG pools.

EPIDEMIOLOGY OF HEPATITIS A AND E

Despite sharing the primary mode of transmission via water contaminated with human feces, HAV and HEV have very different distributions of both disease and infection worldwide. These differences appear to be linked at least partly to the efficiency of virus transmission, with HAV being excreted in very high titers, allowing both waterborne and person-to-person spread. However, unresolved questions remain regarding the prevalence of HEV infection in areas of high endemicity, such as Nepal, where infection with HAV is almost universal, yet less than 50% of the population shows evidence of exposure to HEV (Clayson et al., 1997; Anderson et al., 1999).

Epidemiology of HAV

HAV shows four major patterns of infection worldwide, largely reflecting sanitation standards (Bell et al., 2005). In countries or areas with very poor sanitation, high levels of HAV exposure result in most individuals becoming infected at an early age, but in these circumstances, HAV is not a major public health problem because the symptoms tend to be mild or absent in children and the adult population is no longer susceptible. However, clinical HAV in young children is certainly seen on occasions, most obviously in food-borne outbreaks in schools of developed countries (Reid and Robinson, 1987; Niu et al., 1992; Hutin et al., 1999) but also in populations where HAV is endemic (I. L. Shrestha, personal communication).

Countries with intermediate endemicity are usually those in which rapid improvements in public health and sanitation infrastructure delay exposure to the virus, but the ongoing rate of transmission and potential for common-source outbreaks is still sufficiently high that a significant proportion of adolescents and adults may be exposed with a high risk of disease. The major outbreak in Shanghai in 1988 provided an example of this susceptibility in a country of intermediate endemicity. In countries or populations with high standards of personal and public sanitation, a lower proportion of individuals are exposed at an early age, and thus, the adult population will largely be susceptible to infection. Because of the high transmissibility of HAV, any large population is likely to have ongoing sporadic HAV infection at a low level, causing significant levels of disease in these susceptible adults. The enormous impact of targeted (and now universal) childhood HAV immunization programs on disease incidence in adults (see "Prevention of Waterborne Hepatitis," below) underscores the major role of children in sustaining transmission of HAV in countries with good public sanitation. Finally, countries with the lowest endemicity (such as the Scandinavian countries) have negligible endemic transmission beyond contacts of imported cases.

The public health problem of HAV in some areas of low to intermediate endemicity is further compounded by the potential for large outbreaks of HAV spread by contaminated foods. In the past, such outbreaks were largely confined to shellfish, which by virtue of their filter feeding, are able to concentrate viruses such as HAV from very large volumes of water and are generally eaten raw or only lightly cooked. Even slight fecal contamination of waterways can then lead to large outbreaks of shellfish-associated HAV infection in the susceptible adult population, such as seen in Shanghai in 1988 (Halliday et al., 1991) and in the United States in 1991 (Desenclos et al., 1991). However, the increased transport of fresh and frozen foodstuffs between countries now creates additional sources of infection, such as a number of outbreaks within the United States that have been traced to produce imported from Mexico (Reid and Robinson, 1987; Niu et al., 1992; Hutin et al., 1999). More recently, a large outbreak with 601 patients and 3 deaths was traced to imported green onions served at a single restaurant in Pennsylvania (Wheeler et al., 2005).

The combination of high viral titers in excreta and the high stability of HAV also pose a substantial risk in contamination of groundwater sources. Detailed studies of an outbreak in Canada demonstrated the presence of HAV in wells up to 60 m from the implicated contamination source, and HAV RNA was present for at least 6 months after the first contamination (De Serres et al., 1999).

Tourism also poses a significant risk for HAV infection (and to a lesser extent, HEV infection). For example, a cross-sectional study in Switzerland, which has very low rates of endemic infection, showed that more than 80% of the population over 20 years of age had lifetime risk factors for exposure to HAV (mostly from travel), but with very low vaccination rates (Mohler-Kuo et al., 2007). Intravenous and other illicit drug use is also positively associated with a higher risk of HAV infection and may play a major role in the epidemiology of HAV in some societies (Shaw et al., 1999).

An important aspect in the epidemiology of HAV is the high titer of virus produced, combined with the very great physical stability of the viral particle, which is relatively insensitive to extremes of heat (Anderson, 1987). These factors undoubtedly contribute to the high rate of secondary HAV infection among household contacts and between children. Conversely, HEV has been shown to be somewhat less stable than HAV (Emerson et al., 2005b) and the low rate of person-to-person spread of HEV is likely to be due to a combination of lower particle stability and lower virus titers in excreta than those of HAV.

Epidemiology of HEV

The epidemiology of HEV infection is more complicated and less well understood than that of HAV, in part due to the role of zoonotic strains of virus (see below) but also as a result of the variable sensitivity and specificity of the assays, which have been used to determine seroprevalence (that is, past infection), as IgG responses to different HEV antigens vary widely.

HEV infection is most easily recognized in its epidemic form, occurring every 7 to 10 years in countries in which it is endemic. In general, epidemics are associated with the wet season (summer in many countries in which it is endemic), and the highest clinical attack rate is among young adults. For example, HEV was shown retrospectively to be responsible for 16 of 17 epidemics of enterically transmitted hepatitis in India (Arankalle et al., 1994). In these epidemics, the detection of around 70% serological reactivity among patients (using first-generation assays) is sufficient to implicate HEV as a major cause of the outbreak. The role of HEV in sporadic cases of hepatitis is less well defined, but studies in Nepal and India have shown that HEV is responsible for the majority of acute sporadic hepatitis (Arankalle et al., 1993; Clayson et al., 1997). Serological reactivity to HEV among sporadic hepatitis patients in Nepal during the wet season in 1997 is shown in Fig. 3, implicating HEV in around 70% of cases even though there was no recognized epidemic at that time.

It is also worth noting that outbreaks of HEV can overlap with HAV due to the similarities in their transmission routes; in a study of 33 outbreaks of acute viral hepatitis in Cuba, 14 (42%) were found to have evidence of both HAV and HEV infection, while HAV alone or HEV alone were found in 12 and 7 outbreaks, respectively (Rodriguez Lay Lde et al., 2008).

Early studies detected surprisingly low rates of anti-HEV in countries where epidemics of HEV were known to have occurred in the past and sporadic infection is ongoing. However, it is now clear that these assays failed to detect many individuals in these countries with past infection due to a loss of the antibody against the specific antigens used.

Other serological assays have now been developed that should eventually help to resolve HEV epidemiology by their ability to detect specific IgG in the majority of patients who have recovered from HEV infection (Li et al., 1997b; Zhang et al., 1997; Robinson et al., 1998; Anderson et al., 1999). On a cautionary note, however, there is no consensus regarding the true rate of HEV infection in developed countries, and while reverse transcription (RT)-PCR methods are now well established for diagnosis of HEV infection in reference laboratories (Mushahwar, 2008), their use remains too limited to provide any meaningful estimate of population exposure to the virus. All assays for HEV-specific IgG that have been developed to date detect at least 1 to 2% prevalence in countries in which HEV is presumably not endemic, but very poor concordance has been seen between HEV assays in a study of a serum panel containing many such reactive specimens (Mast et al., 1998), and this lack of concordance appears to be reflected in the disparity between reports of HEV epidemiology. For example, ELISAs based on two different preparations of HEV subviral particles detected anti-HEV in approximately 20% of sera from the United States with no correlation to presumed risk factors (Thomas et al., 1997), and very similar rates of anti-HEV have been reported in a country of endemicity (Saudi Arabia) using these same assays (Ghabrah et al., 1998). In contrast, an ELISA based on the ORF2.1 protein expressed in *Escherichia coli* appears to be effective for detection of past HEV infection in a country of endemicity (Nepal), with almost 33% of individuals reactive, but less than 2% of sera from Australia are positive by this assay (Anderson et al., 1999). This assay detected a much higher prevalence in indigenous communities (presumed to have higher risk factors) than in metropolitan populations in Malaysia (Seow et al., 1999), suggesting that it has considerable utility for measuring HEV seroprevalence.

Even within countries or populations that have potential risk for HEV exposure, the actual rates of exposure can

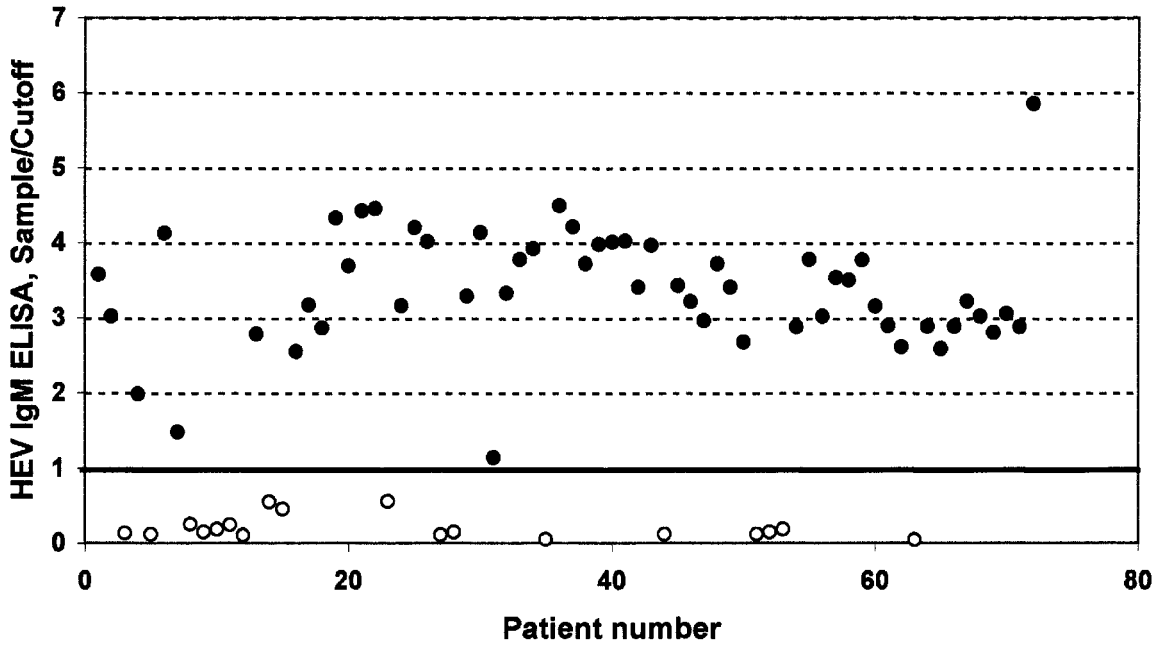

FIGURE 3 HEV IgM reactivities of patients with acute hepatitis in Nepal. Samples from patients with symptoms consistent with acute hepatitis in the month of August 1997 were tested in a prototype HEV IgM ELISA based on the ORF2.1 antigen, at the Siddhi Polyclinic, Nepal. Results are shown as the sample-to-cutoff ratio (data from I. L. Shrestha and D. Anderson).

vary widely. In a study in central Tibet, villages classified as having "poor water quality" on the basis of engineering assessment were prioritized for construction of new water systems. Retrospective studies showed that the average rate of HEV seroprevalence was indeed almost threefold higher in these villages (42.6% versus 13.7% using the ORF2.1 ELISA), but while the average rate of past HEV infection was lower in the villages that were considered to have better water quality, some of these individual villages had rates approaching 50% (Toole et al., 2006). In these situations, HEV seroprevalence may provide an objective long-term indicator of water quality within individual communities (Toole et al., 2006).

Is There a Zoonotic Reservoir of HEV?

HEV was first detected in pigs in the United States (Meng et al., 1997) and has subsequently been shown to be prevalent in swine worldwide as well as in wild deer in Japan (Matsuda et al., 2003; Tei et al., 2003; Sonoda et al., 2004; Takahashi et al., 2004; Masuda et al., 2005; Michitaka et al., 2007). Swine HEV has been transmitted to macaques, and human HEV has been transmitted to swine (Meng et al., 1998; Meng, 2003), although it is likely that not all strains are able to cross species.

Most human isolates of HEV detected in countries of nonendemicity are closely related to local swine HEV strains, as first noted for the prototypic swine HEV and human US-1 strains (Kwo et al., 1997; Meng et al., 1997; Schlauder et al., 1998), and there have been clearly defined cases of zoonotic HEV infection in Japan associated with consumption of HEV-infected pig or deer meat or liver (Matsuda et al., 2003; Tei et al., 2003; Masuda et al., 2005) (see Meng, 2003 for a comprehensive review of zoonotic HEV infection).

There is thus compelling evidence that infection with zoonotic strains of HEV can cause low rates of clinical HEV in countries where conventional spread of human HEV strains does not occur. This reinforces the need for HEV to be considered in the diagnostic criteria for patients in these countries, but wider use of RT-PCR and/or improved serological assays will be required before we gain a clear understanding of the epidemiology of HEV in these countries. In particular, it should be noted that, apart from the food-borne cases in Japan, no epidemiological links have been made between cases of sporadic HEV and contact with swine or other infected animals.

DIAGNOSIS OF HAV AND HEV INFECTION

Correct diagnosis of viral hepatitis depends on reliable assays for infection with each of the viruses, and the choice of which viruses (and thus which assays should be given priority) must take into account the patient history and the likelihood of risk factors for exposure to each virus. In the absence of a relevant travel history to developing countries within the past 6 weeks, HEV is much less likely to be implicated than HAV, and in most settings, HEV would also be considered less likely than HBV and HCV. However, there is no doubt that HEV should now be considered along with HAV (and the blood-borne hepatitis viruses) in the diagnosis of acute hepatitis in developed countries, and exclusion of HEV cannot be made on the lack of travel history alone (Kwo et al., 1997; Schlauder et al., 1998).

Diagnosis of HAV Infection

Diagnosis of HAV infection is very straightforward, with the detection of HAV-specific IgM from a single sample of serum or plasma being highly predictive of acute hepatitis A. HAV has a single, immunodominant antigenic site to which all patients react strongly, and the many diagnostic assays available are based on the detection of IgM reacting with intact (but inactivated) particles of HAV. Assays for total anti-HAV and IgG anti-HAV are also available, but their utility in diagnosis is extremely limited (requiring the detection of rising titers of antibody over a 2- to 4-week period). These assays may be useful, however, for determining patient antibody status prior to the administration of inactivated HAV vaccines, but this is generally not cost-effective (Van Doorslaer et al., 1994).

The technology for detection of HAV-specific IgM is sufficiently well established that there are no marked differences between the diagnostic performance of the laboratory-based testing platforms. Choices of diagnostic assays for HAV may reasonably be made on the basis of convenience for the testing laboratory, with a range from single-strip ELISAs for small numbers of specimens to completely automated systems for high-volume laboratories.

While HAV diagnostic tests are well established, it remains important to restrict testing to patients with a clinical history and/or exposure history consistent with acute hepatitis, because the incidence of hepatitis A infection has declined over the past decade in the United States and other countries as the HAV vaccine is more widely used and all serological assays are prone to at least a low level of false positivity.

Diagnosis of HEV Infection

Diagnosis of HEV infection is less well established, with a number of research and commercial immunoassays available in various countries but with major differences in their sensitivity and specificity (Mast et al., 1998). Prior to the development of comprehensive serological tests, the detection of HEV RNA during viremia provided the only unequivocal evidence for acute HEV infection, with very good sensitivity when performed under ideal conditions (Clayson et al., 1995). This method has also been instrumental in the detection of divergent HEV strains where the serological responses have not been detected by some assays (Hsieh et al., 1998; Schlauder et al., 1998; Wang et al., 1999); however, such detection can only be achieved by sensitive RT-PCR with attendant problems of specificity and specimen transport and handling, exacerbated by the conditions in countries where HEV is endemic. In addition, the period of viremia is short, and RT-PCR is therefore unsatisfactory for routine diagnosis of HEV in most settings but remains important as a diagnostic and reference assay in settings where HEV is not endemic (Mushahwar, 2008).

The appropriate use and interpretation of serological assays for HEV infection must take into account the widely varying prevalence of HEV infection worldwide.

Diagnosis of HEV Infection in Areas of Low Prevalence

In areas of presumed low prevalence of HEV infection, such as the United States and other countries with high levels of sanitation, test specificity will have a very large impact on the predictive value of test results. The detection of HEV-specific IgG formed the basis of first-generation diagnostic assays for HEV (manufactured by Genelabs Diagnostics and Abbott Diagnostics) and had considerable value for the diagnosis of acute hepatitis among travelers who returned from areas where HEV was endemic (Dawson et al., 1992), among whom the incidence may be much higher than the background rate of reactivity. However, around 2% of the healthy population

in areas in which HEV is not endemic are reactive in these assays, and with the recognition that HEV should be considered in the diagnosis of sporadic acute hepatitis without a travel history (Kwo et al., 1997; Schlauder et al., 1998), the need for more specific tests becomes evident. As an example, if the incidence of HEV infection among acute hepatitis patients in the United States was 0.2%, then only 1 in 10 patients reactive in a test for HEV-specific IgG would be true positives. The detection of HEV-specific IgM should therefore become the method of choice for diagnosis of acute HEV infection in areas of low prevalence such as the United States and Western Europe, with RT-PCR providing an important confirmatory assay where available. HEV-specific IgA may also be useful in combination with IgM (Takahashi et al., 2005), but more detailed studies are required.

First-generation HEV IgM assays were based on a cocktail of recombinant ORF2 and ORF3 antigens to increase sensitivity for detection of patient antibody, but the antigens present in these assays still failed to detect around 40% of patients with acute HEV infection (Yarbough et al., 1996a) and the assays had a false-positive rate in the range of 3%.

A number of improved recombinant antigens are now in use in research laboratories, but they have not been widely adopted in commercial tests. The only example currently in commercial use is ORF2.1, representing amino acids 394 to 660 derived from a Chinese strain of HEV expressed in *E. coli* (Li et al., 1994; Li et al., 1997a; Anderson et al., 1999), which has been shown to represent immunodominant and highly conserved epitopes of HEV (Riddell et al., 2000). This antigen forms the basis of a recently developed commercial HEV IgM ELISA (MP Biomedicals Asia Pacific, Singapore, Republic of Singapore), which demonstrated sensitivity of 99.3% (150 of 151 HEV-infected patients) and a false-positive rate of 2.4% (5 of 208 controls) (Chen et al., 2005). The sensitivity of the commercially available ORF2.1-based ELISA therefore appears to be adequate for diagnosis of acute HEV infection, but the likely false-positive rate is around the same as that of previous assays and caution must still be exercised in interpreting positive results from patients without a history of travel outside areas of low endemicity. A number of other commercial HEV IgM assays are available in various countries, but their specificities and sensitivities have not been reported to date.

Diagnosis of HEV Infection in Areas of High Prevalence

While high titers of IgG in patients are suggestive of acute infection, this correlation is imperfect (Ghabrah et al., 1998; Anderson et al., 1999), and the detection of IgG antibody is thus of little use for diagnosis of acute infection in developing countries where HEV is endemic and large numbers of patients will have antibody from past infections. The practical limitations of RT-PCR also preclude its widespread use in these settings, and detection of HEV-specific IgM is therefore the clear method of choice.

As HEV accounts for as much as 70% of the acute sporadic hepatitis in countries in which HEV is endemic, the specificity of assays is less important than in settings in which HEV is not endemic. For example, if the false-positive rate of an assay is 2%, then only 1 in 35 hepatitis patients in areas in which HEV is endemic might be misdiagnosed with acute HEV. Ideally, assay sensitivity should be sufficiently robust to allow assays to be processed and interpreted manually, where equipment such as ELISA washers and readers are not available. In this context, it is worth noting that the original prototype ORF2.1-based IgM ELISA demonstrates very high sample-to-cutoff ratios for the majority of patients (Fig. 3) and this characteristic appears to be retained in the commercial assay.

RPOC Diagnostic Tests for Hepatitis A and E

Rapid, point-of-care (RPOC) tests can have advantages for both clinicians and patients. In view of their common major routes of transmission and relatively good prognosis, the rapid diagnosis of HAV and HEV can quickly determine whether investigations of HBV or HCV are necessary, with the attendant concerns that this can raise in patients.

An RPOC test (Assure HEV IgM; MP Biomedicals Asia Pacific, Singapore, Republic of Singapore) is now available for detection of HEV-specific IgM (Chen et al., 2005; Myint et al., 2005). In the first published study, the RPOC test demonstrated sensitivity of 96.7% (146 of 151 HEV patients) and a false-positive rate of 1.4% (3 of 208 controls), similar to the ORF2.1 ELISA on the same samples (Chen et al., 2005). In a separate study of the Assure HEV IgM RPOC test, where the comparator assay was an in-house quantitative IgM ELISA based on baculovirus-expressed HEV antigen (Seriwatana et al., 2002), the RPOC assay was also found to have a high sensitivity (93%; 186 of 200 HEV patients) and a false-positive rate of 0.3% (1 of 321 controls) (Myint et al., 2005). The RPOC test takes around 5 min in total and requires minimal training and no specialized equipment. Representative assay results with the Assure HEV IgM test are shown in Fig. 4. This assay should prove especially useful in the clinical settings of developed countries, providing a rapid result to guide disease management or the selection of further tests where necessary. In developing countries and refugee settings, these tests offer the further benefits of reduced collection of venous blood (which is not well tolerated in some cultural settings) and rapid identification of outbreaks remote from laboratory facilities. Prototype RPOC assays for HAV have been developed using similar technology (Anderson, 2000) but are not yet commercially available.

PREVENTION OF WATERBORNE HEPATITIS

Due to its enteric transmission, control of waterborne hepatitis on a public health basis relies first on protection of the water supply from contamination with human feces. However, the high rate of person-to-person transmission (especially where children are infected) and relatively common food-borne outbreaks associated with imported fresh and frozen produce contribute to a sustained level of hepatitis A disease in the United States and other developed countries despite the general standards of hygiene in the community.

Passive immunization with human IG was used for many years to provide short-term protection against hepatitis A to travelers and in the control of outbreaks. There are now several highly efficacious, inactivated vaccines available against hepatitis A. Havrix (Glaxo SmithKline, Rixensart, Belgium) and Vaqta (Merck, Whitehouse Station, NJ) have been in clinical use for more than 15 years since registration in most countries, and more recently, Epaxal (Crucell, Leiden, The Netherlands), Avaxim (Sanofi Pasteur, Val de Reuil, France), and Healive (Sinovac, Beijing, People's Republic of China) have become available in various countries. Active vaccination has now largely replaced the use of IG for protection of individuals at high risk of HAV infection. In addition, Twinrix (Glaxo SmithKline, Rixensart, Belgium) is a combined hepatitis A and hepatitis B vaccine that has at least equivalent efficacy for both components in comparison with single vaccines in both children and adults (Van Damme

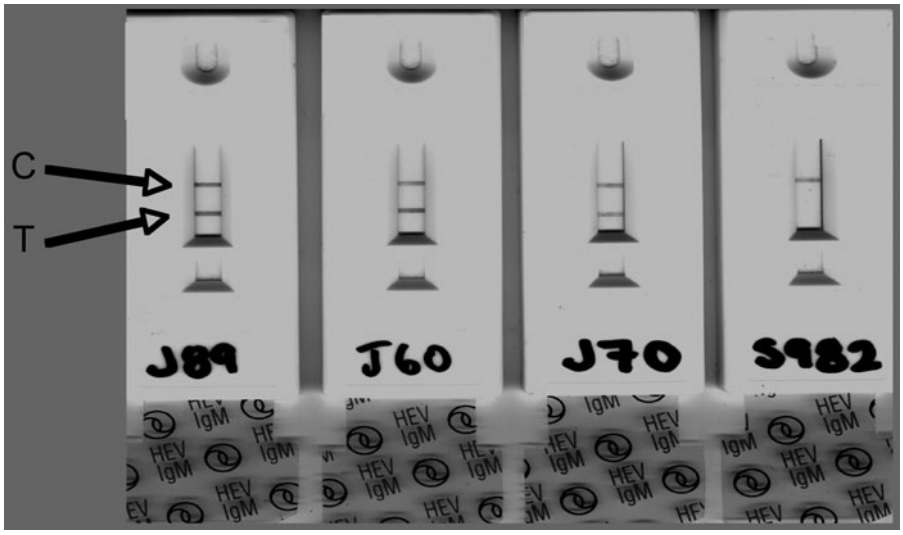

FIGURE 4 Representative test results obtained with the Assure HEV IgM RPOC test. A sample with no detectable HEV-specific IgM (S982) shows a single line (control, C), while samples with HEV-specific IgM (J89, J60, and J70) show both control and test (T) lines. (Reprinted from Anderson and Shrestha, 2009, with permission of the publisher.)

and Van der Wielen, 2001; Van der Wielen et al., 2006). All HAV vaccines have protective efficacies of greater than 95% after 2 doses, with minimal side effects largely confined to local reactions. The very high efficacy of these vaccines, combined with the relatively long incubation period of HAV, suggests that they are also appropriate for intervention in outbreak settings and for household contacts (Sagliocca et al., 1999), although IG is still generally recommended for postexposure prophylaxis where there is no likelihood of future exposure.

In the United States, childhood vaccination against HAV was implemented beginning in 1996 for children in communities with the highest rates of infection (such as Native American and Alaskan Native communities), which led to a 97% reduction in the incidence of HAV infection by 2000 in these communities but had little effect on national infection rates (Bell et al., 2005). The recommendation was extended in 1999 to all children in those counties with a history of high rates of disease incidence (>10 cases per 100,000 in 1987 to 1997) and has led to a >90% reduction in national disease rates, to 1.2 cases per 100,000 in 2006 (Wasley et al., 2008). Implementation of the 2006 recommendation of universal childhood immunization against HAV in the United States is likely to further reduce this incidence, and both Havrix and Epaxal have been shown to be effective in infants at age 12 months (Bell et al., 2007; Van Der Wielen et al., 2007), with only a slight blunting of responses due to maternal antibody in babies at 6 months (Bell et al., 2007). Although the cyclical nature of HAV incidence suggests that caution should be exercised when interpreting the absolute disease incidence based on short-term trends (Bell et al., 2005), there can be no doubt that HAV vaccination in children is an effective public health measure, and it is hoped that other developed countries will implement similar policies in the near future. Countries with intermediate or high levels of HAV transmission may not be able to achieve the same cost-benefit ratios, but the dramatic success of this expanded program in the United States certainly prompts further exploration of its feasibility elsewhere.

Despite the efficacy of HAV vaccines and the likely availability of HEV vaccines in the future, travelers to areas in which these diseases are endemic should be reminded that all precautions should be taken to avoid other waterborne infections, as widespread infection with these hepatitis viruses is a sure indication that water is regularly subjected to contamination with human feces and, therefore, other viruses and bacteria are likely to be present. Only bottled or freshly boiled water should be consumed, and ice, salads, and raw or partly cooked shellfish should be avoided.

There are no licensed vaccines available for prevention of HEV infection, but recent clinical trial data, building primarily on a decade of research by Purcell, Emerson, and colleagues, is very promising. Vaccines based on the PORF2 protein (amino acids 112 to 607 or 660) expressed in insect cells were shown to be highly protective, in animal studies, against a range of isolates (Tsarev et al., 1994; Yarbough et al., 1996b; Tsarev et al., 1997; Zhang et al., 2001; Zhang et al., 2002; Purcell et al., 2003). The recombinant 53-kDa vaccine that has undergone clinical development represents a soluble (non-VLP) form of amino acids 112 to 607, and the recent clinical trial in 1,794 Nepalese army recruits over a median of 804 days demonstrated that the vaccine was highly effective in this population (Shrestha et al., 2007). Seronegative adults received three doses of either the 53-kDa vaccine or placebo at months 0, 1, and 6; hepatitis E developed in 66 of 896 placebo recipients versus 3 of 898 vaccine recipients, with an efficacy of 95.5% (Shrestha et al., 2007).

While these data clearly demonstrate the feasibility of providing at least short-term protection against HEV infection through active vaccination, further studies will be required to establish the duration of protection to establish its likely utility for use in populations of areas where HEV is endemic. The recent development of an ELISA that appears to be specific for the major neutralizing antibody specificity (based on the peptide from amino acids 458 to 607) is likely to provide an important tool for such studies (Zhou et al., 2004).

Although HAV (and potentially HEV) may be partly controlled with vaccines, there is evidence that some cases

of waterborne hepatitis are due to as yet unidentified viral agents. In studies conducted in India and parts of Africa, screening of patients with sporadic hepatitis for serological evidence of HAV, HBV, HCV, and HEV fails to detect a specific cause in around half the cases (Arankalle et al., 1993; Arankalle et al., 1995; Coursaget et al., 1995); however, it is probable that many of these may be acute HEV that were not detected by the assays used. More convincingly, a retrospective study of one outbreak of waterborne hepatitis, on the Indian islands of Andaman in 1987, failed to detect any evidence of HEV; thus, it appears that a novel agent was responsible for that outbreak involving more than 300 patients (Arankalle et al., 1994).

UNUSUAL FEATURES AND FUTURE PROSPECTS

The reason for the high mortality associated with acute HEV during pregnancy remains obscure (Tsarev et al., 1995), and the incidence of zoonotic HEV infection in areas where HEV is both endemic and nonendemic, such as the United States, requires ongoing study. Serological studies of HEV prevalence must be interpreted with caution because of the widely varying sensitivity and specificity of the assays used, and prevalence can also vary greatly between populations within countries. The true incidence of HEV infection in developed countries will most likely not be known without prospective studies of patients with sporadic hepatitis, using the best serological assays (Chen et al., 2005) combined with well-controlled RT-PCR (Mushahwar, 2008). An effective HEV vaccine should become available within the next few years (Shrestha et al., 2007), although measuring the duration of protection and ensuring supply to the most affected countries will undoubtedly present significant challenges.

The dramatic reduction in HAV incidence in the United States as a result of expanded childhood immunization programs should stimulate similar approaches in other countries because HAV will continue to be a major public health problem while the use of vaccines is limited to perceived high-risk individuals. The combined effects of declining antibody prevalence (leading to a larger susceptible population) together with increasing levels of foreign travel may even result in an increasing incidence of clinical HAV in some countries. Food-borne outbreaks, largely associated with imported produce such as shellfish, vegetables, and berries, will continue to be a problem in many countries, but expanded immunization programs can limit the degree of secondary spread from such outbreaks.

CONCLUSIONS

For the patient with acute hepatitis, the major value in specific diagnosis lies in defining both the long-term prognosis once the acute disease has passed and the risk of transmission to partners, family members, and other contacts. In the case of HAV and HEV, a diagnosis provides reassurance that there will be none of the long-term sequelae associated with the blood-borne forms of hepatitis and highlights the importance of hygiene and the use of HAV vaccine or IG in preventing further transmission. However, the diagnosis of HAV and HEV also has important public health benefits, and efforts to control these infections cannot succeed without thorough evaluation of patients with sporadic hepatitis. Hepatitis A is readily diagnosed with the high-quality tests available, and safe, effective vaccines are already available. Hepatitis E must be suspected in patients with acute hepatitis in whom tests for other hepatitis viruses are negative, in view of the clear evidence for zoonotic forms of HEV causing disease in humans in countries including the United States, Japan, and Korea. A better understanding of HEV disease burden and appropriate use of the candidate vaccine in development will require enhanced efforts in surveillance and diagnosis of HEV, taking advantage of improved serological tests with appropriate use of confirmatory nucleic acid testing in countries where HEV is not endemic.

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Blood-Borne Hepatitis Viruses: Hepatitis Viruses B, C, and D and Candidate Agents of Cryptogenetic Hepatitis

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Some hepatotropic viruses of humans do not appear to have developed sufficiently efficient means of host-to-host spread. Possibly as compensation for this limitation, these viruses have a remarkable tendency to persist for many years in their hosts, often for life, and to circulate copiously in the peripheral blood. As a consequence, apparent or inapparent percutaneous exposure to blood or blood products represents a frequent occasion of contagion.

Blood-borne hepatitis viruses include hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis D virus (HDV). In spite of extensive vaccination against HBV in many countries and considerable underreporting, acute or chronic viral hepatitis due to these viruses still ranks among the most frequent reportable infectious diseases throughout the world. In particular, HBV and HCV are known to infect several hundred million people worldwide, thus representing major health and diagnostic issues.

HBV, HCV, and HDV have widely diverse genomic properties and replication strategies. Nonetheless, their pathological effects on the liver are similar and offer no reliable clinical clues about the specific virus producing the alterations. Moreover, many of the infections by these viruses tend to have an insidious onset and extremely irregular courses of disease. As a result, clinicians depend extensively on the diagnostic laboratory for confirming clinical diagnosis of acute or chronic hepatitis, identifying the causative virus, and evaluating disease progression and therapy outcome. Another feature shared by these viruses is that they are not readily cultivable in vitro, which renders virus isolation impractical for diagnostic use. Laboratory diagnosis has, therefore, relied extensively, and still partly does, on immunoassays demonstrating the viral antigens or their corresponding antibodies. However, after the widespread introduction of molecular methods in the clinical virology laboratory, assays that detect, quantify, and characterize the genome of the causing viruses have acquired a key position in both diagnosis and follow-up of viral hepatitis. Molecular assays provide clinically important additional information and are available in the form of commercial user-friendly kits with rapid turnaround times. As will become clear below, they have not only considerably improved patients' management but also partially modified our understanding of the life cycles of blood-borne hepatitis viruses.

SPECTRUM OF DISEASES PRODUCED BY HBV, HCV, AND HDV

As their names imply, blood-borne hepatitis viruses impact mainly on the liver. However, chronically infected persons may also experience extrahepatic diseases with more or less proof of their etiologic relationship.

Acute and Chronic Hepatitis

Primary, acute infections by HBV, HCV, and HDV are often asymptomatic or paucisymptomatic, with influenza-like manifestations and minimal elevation of biochemical liver tests (Table 1). When symptoms of liver damage are clinically evident, they range from mild, anicteric, or self-limiting icteric hepatitis to rare but frequently fatal fulminant hepatitis, with no major differences depending on the causative virus. Variable proportions of acutely infected persons (see individual viruses below) do not resolve the primary infection and become chronically infected (by convention, hepatitis is labeled chronic when it persists longer than 6 months). The clinical manifestations of chronic infection by HBV, HCV, and HDV are also essentially indistinguishable. Again, they vary greatly both in pattern and severity: persistently infected patients may remain relatively asymptomatic for extended periods of time or indefinitely (and are often termed "healthy carriers" or, more appropriately, "inactive carriers") or develop chronic hepatitis with varying degrees of activity and a remittent or stable course, which may eventually lead to hepatic failure (decompensated hepatitis) and cirrhosis after variable but generally long intervals.

The mechanisms of acute and chronic liver damage are only partially understood, but most data converge to indicate that the lesions are not due to cytopathic effects directly produced by the viruses themselves but are largely mediated

TABLE 1 Some biochemical tests of serum for liver function evaluation

Test	Information provided
ALT and aspartate aminotransferase	Degree of hepatocellular injury and necrosis
Gamma-glutamyl transferase	Marker of cholestasis
Alkaline phosphatase	Confirmation of cholestasis
Direct bilirubin	Degree of cholestasis
Albumin, cholinesterase, total cholesterol, prothrombin time, others	Degree of damage to liver protein synthetic activity
Ferritin	Degree of damage to liver storage activity
Immunoglobulin profile	Immune system activation
Hyaluronic acid, apolipoprotein A1, type IV collagen, type III procollagen N peptide, hepatocyte growth factor, others	Extent of liver fibrosis[a]

[a]There is no consensus as yet on the combination of biochemical tests which, associated with platelet numbers, provides the most reliable noninvasive evaluation of liver fibrosis.

by immune attack on viral antigen-expressing hepatocytes. Indeed, when cell-mediated immune responses are feeble, as typically occurs in individuals infected perinatally or receiving immunosuppressive therapies, liver injury is usually less than would be expected from the extent of virus replication. Conversely, in chronically infected patients, abrupt withdrawal of immunosuppressive treatments is frequently followed by a serious deterioration of the clinical and biochemical picture, despite the fact that the levels of viremia may concomitantly decline. On the other hand, chronic HBV and HCV infections of immunocompromised individuals are particularly difficult to cure.

HCC

Individuals chronically infected with HBV and HCV have a greatly increased risk (20 to 300 times, depending on the specific populations considered) of developing hepatocellular carcinoma (HCC). How infection is related to hepatocyte transformation and cancer remains unresolved, but increasing evidence points to a complex interplay of both direct and indirect mechanisms. The tremendous activity of cell death and regeneration, coupled with chronic inflammation, occurring in the infected livers seems to be a major driving force, since cancer develops after 20 to 30 years of infection mostly on a background of liver cirrhosis. By contrast, the HCC found in HBV patients almost universally carries, integrated in the cell genome, fragments of viral DNA that often encode the regulatory protein HBx and that have been implicated as causes of direct insertional mutagenesis and/or of *cis-* or *trans*-activation of cellular cancer-related genes. Although HCV does not integrate, some of its proteins (primarily the core protein but also NS3, NS4B, and NS5A) have been shown to deregulate cellular control and signal transduction and to produce genomic instability. Cofactors possibly involved include dietary aflatoxins, long-term use of ethanol and oral contraceptives, and diabetes (Michielsen et al., 2005).

Extrahepatic Manifestations

As mentioned, chronic infections with blood-borne hepatitis viruses are often associated with a variety of extrahepatic manifestations. These include arthralgias, the vasculitis known as polyarteritis nodosa (more frequently seen in HBV infection), mixed cryoglobulinemia (more frequently seen in HCV infection), dermatological disorders, and glomerulonephritis (either infection). Association with Sjogren's syndrome, porphyria cutanea tarda, splenic lymphoma, and others is less well documented. All of these forms have an unclear pathogenesis. Although they are generally believed to be immune-mediated (immune complex formation and deposition, complement cascade activation, etc.), extrahepatic virus replication also has been invoked. In any case, spontaneous viral clearance and suppression of viral activity by therapy correlate with their resolution.

Coinfections

Because blood-borne hepatitis viruses share common transmission routes, coinfections with two or more of them are not uncommon. Chronic dual HBV-HCV infection is often characterized by low levels of viremia of one or both viruses, by particularly severe clinical and histological presentation, and by poor responses to therapies. Also, acute HCV superinfection of HBV-infected persons may be a major cause of fulminant or subfulminant hepatitis. Since HDV is dependent on HBV for productive replication, dual HDV-HBV infection is the rule and will be discussed below.

Coinfection with one or more hepatitis viruses and human immunodeficiency virus (HIV) is also a frequent occurrence among people at risk of parenteral infection, such as injecting drug users (IDUs), patients on hemodialysis, and individuals practicing unsafe sex. In Western countries, chronic HBV infection affects 5 to 10% of HIV-positive individuals, but this rate is significantly greater in geographic areas in which both infections are endemic at a high level, including Southeast Asia and sub-Saharan Africa. On the other hand, approximately 25% of the HIV patients in the United States also are infected with HCV. These dual infections are currently a matter of great concern due to accelerated progression toward cirrhosis and end-stage liver disease and increased difficulty in treating liver disease. In fact, now that HIV-infected people live longer thanks to highly active antiretroviral drugs, progression to end-stage liver disease has emerged as a leading cause of death among HIV-infected individuals, especially when HIV replication is not well controlled. HBV-HDV-HIV and HBV (HDV)-HCV-HIV coinfections also occur but have been investigated to a limited extent.

Diagnostic Criteria

Diagnosis and severity assessment of acute and chronic hepatitis rely largely on biochemical exploration of liver integrity and function (Table 1). The levels of aspartate aminotransferase and, especially, alanine aminotransferase (ALT) are of

utmost importance, since these enzymes are highly sensitive indicators of hepatocellular damage and provide a simple means of monitoring hepatitis activity. The dynamics of enzyme alterations may even furnish clues about the causative virus, albeit weak ones: e.g., an abrupt, sharp elevation of ALT is considered suggestive of hepatitis A, whereas fluctuating levels are more typical of hepatitis C. Determining the viral loads in plasma or serum provides a more direct assessment of viral replication activity; thus, it has become a precious tool for monitoring the evolution of infection and response to therapies (see below). The extent of hepatic fibrosis has prognostic value for developing cirrhosis and predicts response to therapy (and in HIV-infected patients, risk of hepatotoxicity by antiretroviral drugs). The gold standard for grading liver fibrosis still remains liver biopsy (Strader et al., 2004; Bourliere et al., 2006). This procedure is usually performed before starting antiviral treatments to exclude nonviral causes of liver damage and after treatments to evaluate their efficacy but should be carried out only when indicated and, possibly, during periods of relatively low hepatitis activity. Evaluating characteristics and severity of histological lesions requires expertise outside the scope of the clinical virology laboratory. The virologist can, however, be requested to evaluate the viral content of liver tissue by using immunohistochemistry, gene amplification methods, or in situ hybridization.

Despite its low mortality and morbidity, a liver biopsy is frequently refused by the patient. For this reason, and to avoid the possible sampling errors associated with the procedure, several noninvasive surrogate tools have been proposed in recent years. Of these, the most promising is a panel of serum markers measuring matrix components, enzymes involved in the hepatic fibrosis process, and liver function (Table 1), which is often used in conjunction with the assessment of liver stiffness by elastography. The latter method seems to be particularly accurate in identifying severe liver fibrosis.

CRYPTOGENETIC HEPATITIS AND CANDIDATE VIRUSES

According to a recent estimate (Hsu et al., 2006), the forms of hepatitis clinically indistinguishable from the others that, despite much improvement in diagnostic methods, cannot be ascribed to any currently known pathogen represent approximately 10 to 20% of acute sporadic hepatitis, 5% of chronic hepatitis, occurring frequently in blood-transfused patients, and 10% of fulminant hepatitis cases. Investigation of patients with these cryptogenetic forms of liver disease using a variety of molecular methods aimed at identifying possible causing agents has led to the discovery of a number of novel viruses. Viruses thus unveiled include GB type C virus (GBV-C, also called hepatitis G virus [HGV]), torquetenovirus (TTV), now known to be the prototype of a vast array of viruses classified within the novel genus *Anellovirus*, and others (Yeh et al., 2006). Although the hepatopathogenic potential of these viruses is much less pronounced than originally proposed or possibly nonexistent (they are frequently found to coinfect with the major hepatitis viruses without appreciably impacting clinical conditions), these candidate agents of hepatitis are discussed here because they can be borne by blood and because, for some of them, the ability to injure the liver, alone or in association with other agents, is still under scrutiny.

Below, the viruses considered in this chapter are examined separately because of their distinctive properties. For each virus, a concise summary of the most relevant biopathological features is provided, and the approaches used for diagnosis and follow-up are discussed. Regarding the latter aspect, special emphasis is given to the methods that are most widely used, have entered the clinical laboratory most recently, or only have been proposed but appear suitable for routine use in the near future. For assays that are no longer in common usage, the reader is referred to previous editions of this manual. The procedures for commercial assays are accurately described in the manufacturers' accompanying leaflets and should be strictly followed, while those of in-house methods are too varied to be detailed here.

HBV

HBV is a well-recognized cause of significant liver morbidity and mortality. Although anicteric or even symptomless cases are common especially in children, the clinical course of the infections produced is generally more severe than in other forms of viral hepatitis.

The Virus

HBV was first demonstrated in the 1960s, when the so-called Australia antigen, which had been serendipitously detected in the blood of an aborigine, was linked to what was then mainly known as serum or long-incubation hepatitis. Together with a number of similar mammalian and avian viruses, HBV is classified within the family *Hepadnaviridae*. As such, it is an enveloped virus containing a circular, partially double-stranded DNA genome which uses a reverse transcriptase as a genome-replicating polymerase and an RNA intermediate as a template for the production of progeny genomes. Key structural and replication features of HBV are summarized in Fig. 1 and Table 2. Integration of the viral genome in host cell chromosomes may occur but is not required for virus replication and is a late event in infection often observed in HCCs (Table 2).

Antibodies to the pericapsid proteins, hepatitis B surface antigen (HBsAg) (Table 3), are protective, and based on cross-protection criteria, there is only one serotype of the virus. Fine differences in the antigenic determinants of HBsAg have, however, allowed for the ability to distinguish HBV into immunotypes. These all have in common the *a* determinant, representing the group-specific antigen and containing the major cross-protective neutralization moiety, but differ in other determinants that are present in one of two allelic forms (e.g., *d*-y and *w*-*r*). This results in at least eight different antigenic formulas, the most frequent of which worldwide are *adw*, *adr*, and *ayw*. In more recent years, this antigenic distinction, which had proved useful in epidemiological investigations, has been almost completely replaced by a classification based on genetic relatedness, which appears to have wider implications, possibly including important clinical features (see below). Eight genotypes or clades (designated A to H) have been defined, diverging 8% at least in total DNA sequence (Okamoto et al., 1988; Norder et al. 2004; Devesa and Pujol, 2007) and having specific genomic signatures (Table 4), and most of these have been further distinguished into subgenotypes (nucleotide divergence greater than 4%). Intergenotype recombinants and even mosaic genomes also have been described which are believed to have originated in individuals coinfected with different genotypes, although the molecular mechanisms involved are not understood. In fact, infections with two, occasionally three, distinct HBV genotypes are not rare, occurring at rates of 4 to 17% in different surveys. In addition, phylogenetic

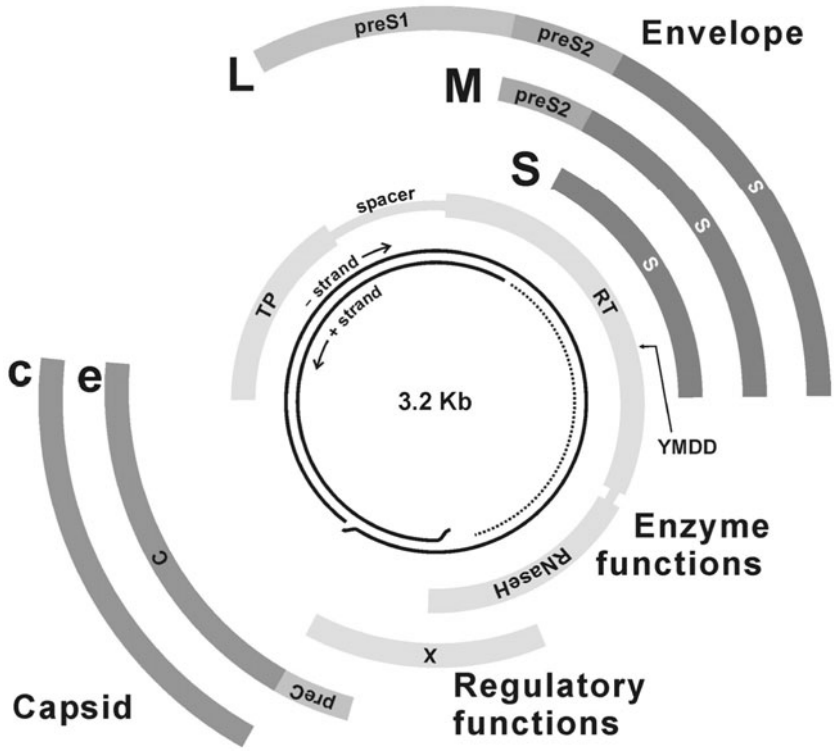

FIGURE 1 Organization of the HBV genome and encoded proteins. The double circle represents the partially duplex DNA genome found within virions and consisting of one nicked complete strand (outer circle) and one incomplete and also nicked strand (inner circle, with the broken line representing the missing segment). However, the functional genome found in infected cells is a cccDNA. The arcs represent the protein products. Abbreviations: c, capsid (core); e, e antigen; L, M, and S, envelope glycoproteins; RT, reverse transcriptase-DNA polymerase; TP, terminal protein. The approximate location in the RT of the YMDD amino acid motif important for resistance to lamivudine is also shown.

analyses have estimated that HBV and one of its closest relatives, the woodchuck hepatitis virus, started to evolve independently about 10,000 years ago and that the immunotypes *adw*, *adr*, and *ayw* separated about 3,000 years ago (Schaefer, 2007).

Due to the high replication capacity of HBV (up to 10^{13} virions enter the blood of infected individuals daily) and the high error rate of its polymerase, it has been calculated that viral genomes with all possible mutations at every nucleotide position are produced every day in infected individuals. Indeed, the HBV isolates obtained from patients can present a variety of mutations, of which those of greater clinical and diagnostic interest include (i) pre-C stop codon mutations, (ii) C promoter mutations, (iii) S gene mutations, and (iv) reverse transcriptase mutations.

Pre-C stop codon mutations (the most common is a G-to-A substitution at nucleotide [nt] 1896) result in an inability to produce the precore protein, i.e., the precursor from which the seromarker of high viral replication HBeAg derives (Table 3). Patients infected with these HBeAg-minus mutants do not present the HBeAg in blood but (different from those with wild-type HBV who have seroconverted to HBeAg negativity) do not tend to resolve the infection. However, these viral variants not only complicate the serological monitoring of patients but may also affect prognosis, being implicated in particularly severe forms of acute and chronic hepatitis as well as in the relapse of apparently resolved infections (Ganem and Prince, 2004; Yim and Lok, 2006; Baumert et al., 2007).

Patients with mutants containing C promoter mutations tend to have a particularly aggressive course of hepatitis most likely due to enhanced viral replication; some also exhibit reduced or indemonstrable levels of HBeAg.

Viral mutants with S gene mutations may present changes in the group-specific determinant *a*, representing the major protective epitope contained in hepatitis B vaccines. Mutants of this kind (escape mutants) have been implicated in a number of cases of hepatitis B that have occurred in vaccinated individuals. They can also be responsible for failures of HBsAg detection in immunoassays to identify infected patients.

Reverse transcriptase mutations may hinder the action of the antivirals that target the viral polymerase, thus significantly affecting patient management (see below). Due to overlapping reading frames of the polymerase and envelope genes, they may also affect the envelope glycoproteins including the *a* determinant (and the opposite is, of course, also true), causing diagnostic problems and failures to protect with vaccination or HBV-specific immunoglobulin (HBIG).

Experimentally, chimpanzees are readily infected by HBV and exhibit an infection course similar to that of humans. HBV replication in vitro has proven hard to achieve. Limited propagation has been obtained in primary cultures of human hepatocytes. Transfected cell lines are also widely

TABLE 2 Structural, replication, and integration properties of HBV: salient features

Structure
- Spherical, 40- to 45-nm-diameter virion, also referred to as Dane particle
- Icosahedral, 30- to 34-nm-diameter capsid, or core, composed of numerous copies of a single protein (core antigen or HBcAg) (Table 3)
- Tight envelope, composed of three virus-encoded glycoproteins (surface antigen or HBsAg) (Table 3) and cell-derived lipids
- Relaxed, circular 3.2-kb double-stranded DNA; the minus strand is complete and nicked; the plus strand, also nicked, is incomplete for a variably long stretch
- Genome organized in four extensively overlapping ORFs: pre-S-S (surface glycoproteins), preC-C (core and precore proteins), P (reverse transcriptase-DNA polymerase, RNase H, and terminal genomic protein), and X (HBx, multifunctional protein that activates a wide range of viral and cellular regulatory elements)
- Enzymatic activities packaged in virion: reverse transcriptase-DNA polymerase and RNase H

Replication
- Entry into cells probably mediated by attachment of the viral L protein to unknown cellular receptor(s)
- The partially duplex virion released from the capsid, converted into an episome-like cccDNA, and transported to the nucleus
- cccDNA transcribed by cellular RNA polymerase II to produce multiple copies of a full-length pregenomic RNA intermediate and of subgenomic mRNA
- Pregenomic RNA intermediate packaged in the citosol into a procapsid structure, together with reverse transcriptase, and reverse transcribed into the minus DNA strand, on which an incomplete plus DNA strand is then synthesized
- Nucleocapsids mature, acquire the envelope by budding through the endoplasmic reticulum at sites where viral glycoproteins have accumulated, and are released as complete virions
- No overt cytopathic effects

Integration and HCC
- In many long-term chronically infected individuals and in roughly 90% of HBV-positive HCCs, the hepatocytes contain integrated viral DNA, often in multiple copies
- Integration not necessary for HBV replication
- Integrated genomes are often defective due to deletions or rearrangements, hence reactivation is very unlikely
- Mechanism of integration poorly understood; the virus does not encode for integration machinery, and integration occurs at many different locations in the chromosome often within or near fragile sites
- Transcription of integrated DNA restricted to some subgenomic mRNA, among which the one encoding HBx is often represented
- Role in HCC generation uncertain; the fact that some HCCs lack integrated HBV has suggested a "hit-and-run" mechanism of genetic instability, leading to carcinogenesis during liver regeneration

used for the study of viral biosyntheses. For more comprehensive recent reviews of these and other aspects of HBV, see Gerlich and Kann (2005), Feitelson and Lee (2007), and Seeger et al. (2007).

Dynamics of HBV Replication in Infected Patients and Antiviral Immune Responses

In infected humans and chimpanzees, HBV replicates primarily in human hepatocytes although viral genomes, and replicative intermediates, and other footprints of virus replication have repeatedly been detected in extrahepatic sites, including bile duct epithelium, pancreas and kidney cells, lymphocytes, and monocytes. The bulk of information about the dynamics of HBV infection has been collected by monitoring the levels of virus in the peripheral blood, measured initially as the amount of viral antigens and currently as the copy number of viral genomes. The most sensitive DNA amplification assays, with their high sensitivity, have actually somewhat modified the picture of the natural course of infection and disease and, in particular, have led us to realize that patients who appear to have eradicated the infection when tested with immunological or poorly sensitive molecular methods, may in fact, continue to harbor the virus for long periods of time.

TABLE 3 Major viral proteins exploited for the laboratory diagnosis of HBV infection

Function (current name)	Properties
Envelope glycoproteins or surface antigen (HBsAg)	The bulk of the immunoreactive HBsAg found in plasma is the S glycoprotein (24–27 kDa). HBV virions and subviral aggregates also contain small amounts of two larger products of the pre-S-S ORF known as middle (M) and large (L) pre-S proteins (31 and 39 kDa, respectively) that result from initiation of translation at different AUG codons (Fig. 1).
Capsid protein or core antigen (HBcAg) Truncated capsid protein or e antigen (HBeAg)	Both of these proteins are encoded by the ORF preC-C, which has two translation initiation codons (Fig. 1). Initiation from one codon leads to production of HBcAg, which is incorporated into virions. Initiation from the alternate codon produces the precore protein, which is instead targeted into the cell's secretory pathway. Here, the precore protein is processed and eventually released in the serum of infected patients, where it represents the HBeAg used in laboratory diagnosis as a useful seromarker for active viral replication.

TABLE 4 HBV genotypes: genome differences of prototype isolates and geographic distributions

Genotype	Genome length (bp) (signature[s])	Subgenotype(s); geographic distribution
A	3,221 (insertion of aa[a] 153 and 154 in C)	A1; Africa, Asia, South America A2; Europe, North America A3, A4, A5; Africa
B	3,215	B1; Japan B2, B3, B4, B5; Southeast Asia
C	3,215	C1, C5; Southeast Asia C2; Far East Asia C3; Micronesia C4; Australia
D	3,182 (deletion of aa 1 to 11 in pre-S)	D1; Eastern Europe, North America D2, D5; India D3; South Africa, East India, Serbia D4; Australia
E	3,212 (deletion of aa 11 in pre-S)	West Africa
F	3,215	F1; South and Central America F2, F3, F4; South America
G	3,248 (insertion of 12 aa in HBc, deletion of aa 11 in pre-S1)	Mexico
H	3,215	Central and North America

[a]aa, amino acids.

The possible courses and phases of HBV infection have been variously classified (Chisari, 2000; Ganem and Prince, 2004; Yim and Lok, 2006). From the clinical virology standpoint, however, it seems useful to limit ourselves to distinguishing between self-limited acute infections and those that instead persist longer and become chronic. As already mentioned, hepatitis is considered chronic when it does not resolve within 6 months from onset.

Self-Limited Acute Infections

The incubation period of acute hepatitis B is usually 1.5 to 3 months but sometimes considerably longer. The symptoms associated (malaise, nausea, anorexia, low-grade fever, jaundice, dark urine, and pale stools) usually last 2 to 3 weeks but may be much more protracted. Typically, viremia becomes detectable 3 to 5 weeks before the development of clinical symptoms (i.e., 2 to 6 weeks before the onset of ALT abnormalities) and peaks soon thereafter (Fig. 2A). Complete, infectious 40- to 45-nm-diameter virions (also known as Dane particles) circulate abundantly in the plasma of infected individuals, frequently reaching levels of 10^8/ml or greater, a feature that is reflected in the positivity of all the diagnostic assays that detect viral molecules (HBV DNA, viral polymerase, HBsAg, and HBeAg). However, HBV is unusual among human viruses in that the plasma of patients also contains a large excess of HBsAg proteins that do not participate in the formation of complete virions but are instead aggregated in spherical (16 to 25 nm in diameter) and filamentous (20 nm in diameter and of variable length) lipoprotein structures. These subvirion particles, which are noninfectious due to the absence of the viral genome, are even more abundant (up to 10^6 times) than complete virions. As a result, the blood contains an extremely high concentration of HBsAg (on the order of 50 to 300 μg/ml), which explains why this antigen is such a sensitive seromarker for identifying infected persons (see below).

Nonspecific effectors of antiviral resistance, including interferons (IFN), natural killer cells, cytokines, etc., are believed to represent the first line of resistance against HBV infection. Microarray analyses of liver RNA in experimentally infected chimpanzees have, however, shown that expression of cellular genes remains essentially unchanged during HBV entry and early expansion in the liver. Thus, cytotoxic T lymphocytes (CTL) and other effectors of the adaptive cell-mediated immune responses, such as the antiviral cytokines, likely represent the most important line of defense. Numerous CTL, specific for HBV proteins expressed on infected hepatocytes, are recruited in the liver and probably act in concert with the various types of IFN, as suggested by findings showing that large numbers of IFN-γ-regulated genes are induced during viral clearance. Thanks to their ability to enhance major histocompatibility complex class I protein expression, IFN are indeed known to render infected hepatocytes more vulnerable to the cytolytic action of CTL (under normal conditions, hepatocytes display little major histocompatibility complex class I protein on the cell surface). Studies have shown that the CTL response is especially broadly specific and maximal in those acutely infected patients who resolve the infection. These and other findings have demonstrated the impact of adaptive T-cell responses in terminating the infection but also have revealed that they are major contributors to the generation of hepatic damage (Chang, 2007; Ciupe et al., 2007). Indeed, a state of partial or split immunotolerance is often invoked to explain why many infections remain asymptomatic with little histological evidence of liver injury. In experimentally infected chimpanzees, whereas CD4 T-lymphocyte depletion did not significantly alter the course of infection, CD8 T-lymphocyte depletion markedly reduced liver disease, greatly prolonged the duration of infection, and delayed the onset of viral clearance until virus-specific CD8 T-lymphocytes reappeared in the circulation (Thimme et al., 2003). As discussed below, in humans, subclinical infections are especially numerous when the immune system is poorly reactive (e.g., neonatal age or other causes).

Anti-HBV humoral immune responses are believed to be of less importance in deciding the outcome of HBV infection but are certainly of paramount importance for its

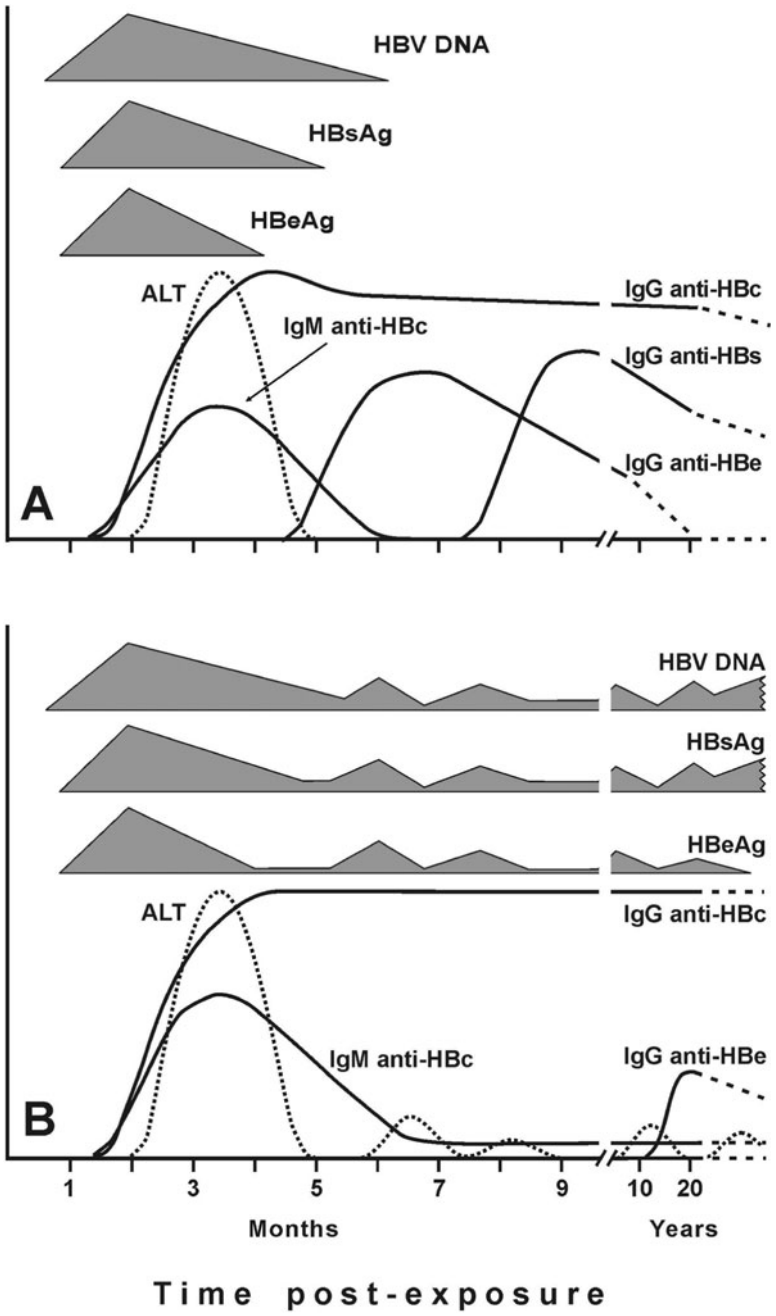

FIGURE 2 Typical courses of HBV infection. (A) Acute self-limited infection. (B) Infection that does not resolve and becomes chronic. While the virological and immunological events depicted are seen in virtually all patients, clinical symptoms develop in a proportion of infected individuals that varies greatly with age at infection and other parameters (see text). The shaded areas represent the periods during which the markers indicated are demonstrable in the circulation. As described in the text, in an undefined proportion of patients recovered from acute self-limited infection, minute amounts of HBV DNA are demonstrable by sensitive gene amplification techniques well beyond the time periods indicated.

identification in the laboratory, since this is usually asked to confirm the clinical suspect after the patients have already gone through the initial preserological phase, characterized by high levels of viremia and absence of antiviral antibodies. Anti-hepatitis B core (anti-HBc) immunoglobulin M (IgM) antibodies are the first antibodies to appear. They typically develop at the same time as ALT changes, increase rapidly to reach considerable titers, and are then progressively, nearly completely, replaced by high titers of anti-HBc IgG (Fig. 2A).

Most likely as a result of the mounting immune response, acute-phase viremia levels undergo a first decline concomitant

with the appearance of clinical symptoms. Subsequently, plasma viremia continues to decline and becomes undetectable, both serologically (HBsAg) and by not-so-sensitive molecular methods (HBV DNA), in a few weeks. Importantly, this phase is usually preceded by the disappearance of HBeAg from the blood and the appearance of anti-HBe antibodies (Fig. 2A). Concomitant with or subsequent to viremia termination, hepatic histology, biochemical parameters, and clinical conditions normalize. Anti-HBs antibodies, which are particularly important because they are capable of neutralizing the virus, also develop and become measurable just before or, more often, a few weeks after the disappearance of HBsAg from the serum. Formerly, this series of events accompanying clinical recovery was considered to invariably correspond to a complete eradication of the infecting virus. However, duplicating what is observed in occult chronic HBV infections (see below), the most sensitive viral genome detection methods have led to the realization that, in some patients, recovery from self-limited acute infection is followed by the persistence of low numbers of viral genomes in plasma for months or years (Akahane et al., 2002). In any case, anti-HBc antibodies persist for life in the great majority of patients (>90%), while anti-HBs antibodies have a somewhat greater tendency to become undetectable with passing years (Fig. 2A).

Chronic Infections

The proportion of acute infections that progress to chronicity (Fig. 2B) depends on a number of variables, including the dose of the viral inoculum, the route of transmission, and the patient's age and immune responsiveness (Yim and Lok, 2006). Of these, age at infection is by far the most important, likely as a result of differences in the ways the virus is confronted by the host's immune responses. Indeed, individuals acquiring the infection perinatally become chronically infected in greater than 90% of cases versus 25 to 30% in children less than 5 years of age and fewer than 10% in adulthood (Slowik and Jhaveri, 2005).

Notably, the primary infections that remain asymptomatic or have a mild clinical course tend to become chronic more frequently than others. Practically, however, failure to resolve the infection due to HBV persistence is revealed only by the observation that viremia does not tend to subside. In fact, chronically infected individuals are characterized by the persistence of markers of active virus replication (HBsAg, HBeAg, and HBV DNA) at levels that remain for years in the same range or slightly lower than in the post-acute phase of the infection. The clinical impact of chronic HBV infections is variable, ranging from a completely asymptomatic state to a progressive chronic hepatitis, potentially evolving to cirrhosis and HCC. Most of the so-called inactive virus carriers are HBsAg and anti-HBe positive in plasma but have normal liver enzymes. In these patients, HBV DNA levels may be low or nearly undetectable, and liver damage ranges from mild inflammation to minimal fibrosis or inactive cirrhosis at most. Importantly, patients with symptoms of hepatitis can alternate clinical manifestations with variably long periods of remission, and the proportion of individuals who progress to cirrhosis and HCC is roughly proportional to disease activity (length, number, and severity of flares). Their viremia is relatively stable over time or, more often, fluctuates in association with fluctuations in ALT and anti-HBc IgM levels (Fig. 2A).

The availability of drugs that effectively inhibit HBV replication has permitted estimates of HBV dynamics in chronically infected individuals. Thus, it has been calculated that, in chronic infections, the virions released into the circulation daily are on the order of 10^{11} to 10^{13} and, although the minimum half-life of infected hepatocytes is relatively long (10 to 100 days), most probably as result of the low cytopathogenicity of HBV, the hepatocytes destroyed and renewed per day are 10^{9} or more. In any case, with passing years of persistence, the levels of infectious virus present in the blood tend to slowly decline, as revealed by decreasing concentrations of HBeAg and HBV DNA. Eventually, usually after many years of HBV persistence, the HBeAg may become undetectable, and this is generally followed by seroconversion to anti-HBe. When resolution occurs, virological and serological markers evolve with a pattern similar to that described for resolution of acute infections, albeit at a slower pace. Spontaneous recovery from chronic infection is, however, infrequent, occurring at rates of only 0.5% per year, and in the great majority of cases, the levels of circulating HBsAg remain essentially unchanged for decades or for life (Fig. 2B).

It is, however, important to emphasize that variations to the above pattern exist. One possibility that should constantly be kept in mind, especially in certain geographical areas, is that patients can become HBeAg negative and anti-HBe positive not because they are in the process of resolving the infection but because they harbor an HBeAg-minus variant of the virus. This situation, which was once considered infrequent outside the Mediterranean basin, is now known to be distributed worldwide, albeit with different incidences. According to recent estimates, HBeAg-minus HBV accounts for over 30% of total forms of chronic hepatitis B in Mediterranean countries and for roughly 15% of cases in North America, Northern Europe, and Asia-Pacific countries (Funk et al., 2002). Progression to this status is either spontaneous or facilitated by immune suppression, and long-term prognosis is generally poorer than that observed with wild-type HBV.

Occult HBV infection, defined as the presence of HBV DNA in the liver and blood in the absence of detectable HBsAg, and very occasionally in the absence of other seromarkers of infection as well, has been recognized as a further form of chronic infection relatively recently through the unmatched sensitivity of current DNA amplification methods. This condition has been found to be particularly frequent in certain high-risk groups, such as HIV type 1 (HIV-1)- and HCV-infected patients (Shire et al., 2007), but also has been detected in blood donors and other groups. Occult infections were initially thought to be caused by replication-defective HBV, but subsequent detection of viral RNA transcripts and covalently closed completely double-stranded DNA (cccDNA) in liver biopsy specimens of many patients, together with observations that the infection is transmissible, have resulted in the belief that they are sustained by wild-type viruses replicating at a low level (Torbenson and Thomas, 2002). However, a large proportion of the viruses involved (up to 40% in some studies) may escape immune detection, as they contain S gene mutations and express low levels and/or antigenically altered forms of HBsAg. Although it is generally accepted that occult hepatitis B can worsen the prognosis of HCV infection, its clinical consequences, including the possible risk of developing cirrhosis and HCC, are still obscure (Chemin and Trepo, 2005).

Epidemiology

The number of HBV-infected people worldwide is estimated at nearly 400 million, with about 50 million new infections annually. Although pockets of comparatively higher

endemicity are found within any geographic area in specific ethnic, behavioral, and professional groups, prevalence rates vary widely in different geographic regions, ranging over 10% in certain areas of sub-Saharan Africa, China, and Southeast Asia and being as low as 0.1 to 0.5% in Western Europe and North America. Similarly, the distribution of HBV genotypes varies widely depending on geography and ethnicity (Table 4). Genotype distribution as well as their further distinction into subgenotypes is, however, dynamic and subject to change as new HBV isolates are sequenced (Custer et al., 2004).

Infected humans are the only source of contagion. In the past, hepatitis B was a frequent complication of blood transfusions and of the therapeutic use of blood products, but it is now a rare occurrence in these clinical settings as a result of specific screening of donations, virucidal treatment of blood derivatives, and other preventive measures. Due to high virus content, minute traces of infected blood suffice for transmission; thus, improperly shared usage of, and incidental injuries with, syringes, needles, medical and toilet instruments, etc., represent other important occasions of contagion. HBV is also present in semen, vaginal and menstrual secretions, saliva, urine, tears, and breast milk, albeit at much lower titers than in blood. In any case, sexual intercourse is a frequent route of dissemination, as shown by the observation that in areas of low endemicity, the highest rates of infection are found in sexually active young adults, with peaks among male homosexuals.

Vertical transmission from HBV-positive mothers to babies is also frequently observed (in the absence of proper immunoprophylaxis [see below], nearly 90% of children born to HBeAg-positive mothers become infected) and represents a major mechanism of virus spread especially in areas of hyperendemicity. The fact that the babies become HBsAg positive only 1 to 3 months after birth and other evidence indicate that transmission occurs during parturition or perinatally rather than in utero. Intrafamilial spread with no apparent vertical, parenteral, or sexual exposure also occurs and appears to be especially important for young children living in areas of high endemicity or institutions. Other modes of transmission are possible but epidemiologically unimportant. For example, experiments have shown that oral transmission is feasible but requires large inocula. However, in developed countries, over one-fourth of infected individuals do not have, or do not report, high-risk behaviors or events (Kao and Chen, 2002).

Prevention and Treatment

HBV is rapidly destroyed by acids and lipid solvents but is relatively heat stable and may retain its infectivity in drying blood and other biological fluids for several days (Gerlich and Kann, 2005). Intervention strategies for limiting HBV dissemination include reduced use of nonautologous blood transfusions, accurate selection of blood and organ donors, proper education of health care workers and other high-risk subjects, decontamination of high-risk environments, administration of HBIG, chemoprophylaxis, and vaccination. Prevention of occupational and nosocomial transmission is essentially based on the presumption that any person might carry the virus and on consequent strict implementation and maintenance of infection control practices. Changes in risk behavior of IDUs should also be pursued. In an attempt to prevent less common modes of transmission, HBV-negative sexual partners and household contacts of virus carriers should be informed about how to reduce the risk of becoming infected (vaccination is of primary importance), while infected individuals should be counseled on how they can prevent transmission of the virus to others as well as on secondary prevention practices that can reduce the risk of progressing toward increasingly severe liver pathology. In particular, HBV carriers should be advised to vaccinate against hepatitis A, to keep away from the risk of becoming superinfected with other hepatitis viruses and HIV, to refrain from drinking ethanol, and to seek appropriate medical care.

The administration of high-titer HBIG consistently, albeit transiently, decreases the risk of acquiring the infection and the severity of hepatitis but, given the high efficacy of vaccination for preexposure prophylaxis, should be limited to postexposure prophylaxis. Combined immunoprophylaxis at birth with both HBIG and vaccination for babies born to HBV-positive women (which should be regarded as postexposure prophylaxis) is especially important and highly effective. Treatment of expecting mothers with selected HBV polymerase inhibitors (see below) during late pregnancy also has been advocated as a means of reducing the risk of HBV transmission to the babies (van Zonneveld et al., 2003).

The first anti-HBV vaccine was developed in the early 1970s and was composed of highly purified HBsAg harvested from infected human plasma and processed to remove or inactivate any infectivity. This immunogen was, however, soon replaced by the recombinant HBsAg vaccines currently in use. Although vaccines have been extensively used with excellent safety and efficacy records (protection lasts for at least 15 years [McMahon et al., 2005]), further improvements have been proposed. Among these, the incorporation of highly conserved pre-S peptides has been proposed for protecting those who do not respond adequately to the standard vaccines. In many countries, routine vaccination has been successfully integrated into the childhood immunization schedule. The incidence of hepatitis B remains, however, high in the adult population, which accounted for approximately 95% of the estimated 51,000 new HBV infections that occurred in the United States in 2005 (Centers for Disease Control and Prevention, 2006). Importantly, information is also clearly emerging that vaccinated individuals have a reduced incidence of HCC as well as of HBV infections, indicating that the HBV vaccine may, in fact, be the first anti-tumor vaccine entered into human use (Chang, 2007).

The anti-HBV agents currently available are efficient at suppressing HBV replication but much less so at eradicating the virus. They include standard IFN-α and pegylated IFN-α (pegIFN-α), which appear to act through their ability to modulate the host's immune response, and several nucleoside-nucleotide analogs, such as lamivudine, adefovir, tenofovir, entecavir, and telbivudine, which target the viral polymerase. Although many patients are empirically treated, the efficiency of antivirals in the setting of acute hepatitis B is essentially unknown. Treatment guidelines for chronic HBV infection are continuously evolving. The main goals are to induce remissions, prevent flares and the development of liver failure, lessen the long-term risk of cirrhosis and HCC, and at the same time, reduce the infectivity of virus carriers. A complete response consists in the seroconversion from HBsAg positive to anti-HBs positive. Although this is obviously a desired therapeutic endpoint, a decline of HBV DNA load in plasma to less than 20,000 IU/ml and/or HBeAg seroconversion are regarded as successful, as they are generally accompanied by a significant improvement in liver disease (Tillman, 2007). A reduction of HBV DNA in blood below 10^4 or 10^3 copies/ml that lasts for at least 24 weeks after therapy has been suspended is generally labeled sustained virological response (SVR).

Although there are indications that antivirals can be beneficial (reduced risk of developing HCC [Chen et al., 2006]) also in the chronically infected patients who have no or minimal signs of liver damage, it is generally agreed that, unless they carry HBeAg-minus HBV variants, these patients should not be treated but examined every 3 to 6 months for possible reactivations of viral replication and ALT exacerbations. In fact, the decision to initiate treatment must be weighed against issues in cost, compliance, and risk of drug resistance development. Thus, treatment is generally restricted to the chronically infected who present active compensated liver disease. A 16- to 32-week course of IFN-α or pegIFN-α is generally considered the first-line option in the HBeAg-positive patients who present predictive factors of favorable response (see below), but in the others, long-term therapy is usually necessary using these or other drugs. The polymerase inhibitors have been shown to induce HBeAg seroconversion in 10 to 20% of patients after 1 year of treatment and in approximately 30% after 2 years but do not usually lead to clearance of HBsAg. Complete response is instead observed in approximately 10% of the patients given IFN-α, a rate of cure three to four times higher than seen in untreated controls monitored for similar periods of time (Lok and McMahon, 2004).

The HBV polymerase inhibitors have fewer side effects and are better tolerated and less costly than IFN-α but, different from this drug, lead to the emergence of drug-resistant virus mutants, a drawback that is especially pronounced with lamivudine (resistance is observed in roughly 30% of patients after 1 year of monotherapy, in almost all with more protracted treatments, and has a particularly early onset in those infected with HBeAg-minus variants [Thompson et al., 2007]). Though in some patients the emergence of lamivudine-resistant variants was associated with worsening of liver disease (Dienstag et al., 2003), virus replication of the drug-resistant viruses generally remains at moderate levels, probably due to a reduced virus replication rate resulting from a weakened affinity of the mutated viral polymerase for natural nucleotides. Adefovir is active against lamivudine-resistant mutants and has a higher genetic barrier to resistance relative to lamivudine (resistance is observed in 1 to 2% of patients after 1 year of monotherapy and in 30% after 5 years) but is more costly, and the durability of response and long-term safety have not been fully determined (Lok and McMahon, 2004). Since resistance to lamivudine may increase the risk of resistance to almost all the other polymerase inhibitors, it is generally advised that lamivudine monotherapy should be avoided as initial therapy. By contrast, the current approach to the management of HBV drug resistance involves the substitution (or increasingly, the addition) of a second antiviral agent to the treatment regimen. Several combination therapies as well as other antiviral agents are under evaluation for efficacy and ability to prevent drug resistance in large-scale clinical trials (Craxi et al., 2006; Mohanty et al., 2006).

Currently, liver transplantation is the only available treatment for decompensated cirrhosis and HCC. However, in HBV-infected patients, this treatment is very commonly followed by the recurrence of infection in the grafted liver and by a particularly aggressive course of the ensuing hepatitis due to immunosuppression (steroids are known to activate HBV expression). The presence of HBV DNA and HBeAg in the prospective recipient is considered highly predictive of posttransplant recurrence (Chung et al., 2001). The drugs discussed above are being evaluated also in the posttransplant context, alone, in various combinations, and in association with HBIG, with some initial success (Gish and McCashland, 2006). Therapeutic infusion of autologous CTL expanded in vivo also has been proposed.

Indicators Predictive of Beneficial Response to Antiviral Treatments

Because HBV therapies may have side effects and are costly, being able to predict whether or not the infection or at least the disease of a given patient will respond favorably would have great practical utility. Clinical trials have shown that serum ALT levels of >100 IU/ml are always predictive of an improved likelihood of obtaining an SVR and that low viral load ($<10^7$ DNA copies/ml), severe inflammation in the liver at biopsy, and a short duration of infection are predictive of a favorable response to IFN-α (Janssen et al., 2005; ter Borg et al., 2006). There is also initial evidence that some HBV genotypes respond better to IFN-α than others. In particular, HBsAg seroconversion seems to be especially associated with genotype A. In contrast, genotypes do not appear to be relevant for response to nucleoside analogs (Tillman, 2007).

Laboratory Diagnosis and Follow-Up

Laboratory diagnosis and staging of HBV infection are achieved through a complex series of immunological and molecular assays. An algorithm guiding their use can be found in Fig. 3, while Table 5 provides a key to the interpretation of test results.

Tests for HBV Antigens and Antibodies

Laboratory diagnosis of HBV infection is generally first obtained by using immunoassays that demonstrate the presence in the patient's serum of specific viral proteins (Table 3) or of the corresponding antibodies. The assays currently most used are commercial enzyme immunoassays (EIA) of various formats and are generally characterized by excellent specificity and sensitivity.

Immunologically, the key marker for diagnosis of infection is HBsAg, which becomes demonstrable in serum 2 to 6 weeks prior to biochemical evidence of liver damage and remains positive throughout the course of acute and chronic infection, except possibly in their very late stages. The lower limit of sensitivity of the assays currently in use is 0.1 to 2 ng/ml serum. When this marker is negative, one can exclude an active infection, except for a very few instances in which the amounts of antigen produced are too small for consistent detectability, as may occur in fulminant liver failure, in neonatal infections, or when HBV is mutated in the *a* determinant of the HBsAg in a way that it is no longer recognized by the antibodies used in the detection assay (Coleman, 2006). When the HBsAg test result is positive, one can proceed to test for the presence of antibodies to HBcAg, which are confirmatory for the diagnosis of infection. Usually, anti-HBc IgM appears 2 to 4 weeks after the HBsAg and, unless the assay method is particularly sensitive, becomes undetectable in a few months, but it is important to keep in mind that low levels can persist for much longer and can reappear or increase in titer (>10 IU/ml) when viral replication increases. Thus, a reactivation of viral replication can be revealed by quantitative determination of anti-HBc IgM. Alternatively, one can proceed to test the serum for the presence and/or titer of HBV DNA with the molecular assays described below.

Additional seromarkers useful for establishing the activity of infection include HBeAg, anti-HBe, and anti-HBs. With the exclusion of infections produced by HBeAg-minus

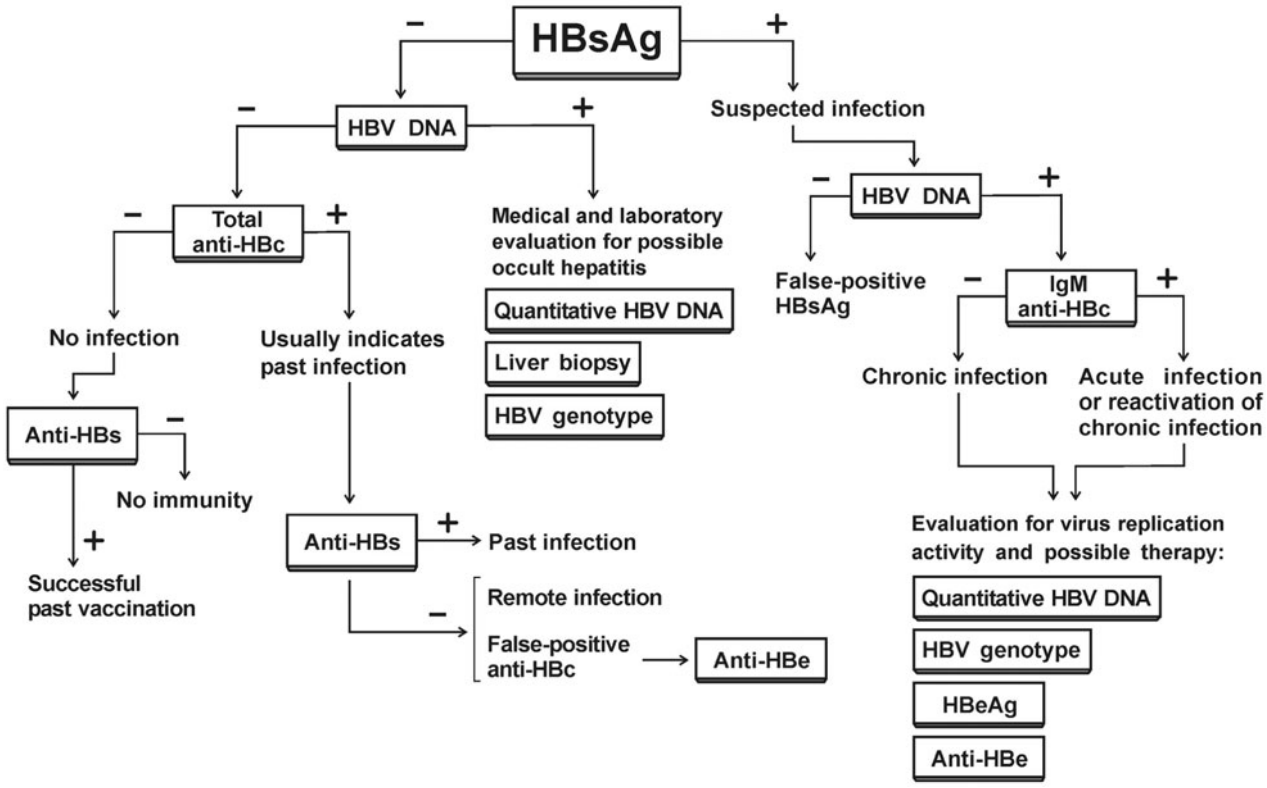

FIGURE 3 Algorithm for use and interpretation of laboratory tests for HBV infection (Table 5). Note that exceptions to the diagnostic conclusions indicated exist and that, especially in certain geographic areas, all HBV infections should also be tested for HDV.

mutants, the HBeAg is found in the patient's blood when the virus is replicating at a high rate, and its continuous presence therefore correlates with active hepatitis. Conversely, disappearance of this antigen and appearance of the corresponding antibodies is indicative of reduced viral replication. In acute infections, these events usually herald resolution, since they are usually followed, within weeks or months, by the disappearance of the HBsAg from the serum and, with a lag of weeks, by the development of anti-HBs antibodies.

Tests for the Detection and Measurement of HBV DNA in Plasma

The immunoassays discussed above permit an accurate diagnosis of most acute and chronic HBV infections. Determining the presence and amount of HBV DNA in plasma is of the utmost utility for patient management and, in selected cases, also for the correct interpretation of immunological data (Fig. 3 and Table 5). Although HBeAg still serves as an important marker for monitoring infection activity (with the exception of the infections sustained by HBeAg-minus variants of HBV, it is generally detectable only in patients who circulate HBV DNA), a negative assay result and seroconversion to anti-HBe does not exclude viral replication (Peng et al., 2005).

In essence, viral DNA detection based on the current gene amplification methods must be regarded as the most accurate indicator of HBV replication, since these assays can reproducibly reveal the presence of very few genomes. In contrast, the commercial hybridization assays that were extensively used in the past had lower detection limits, on the order of 10^5 genomes/ml (Zeuzem, 2004). Thus, detection of HBV DNA by molecular amplification methods in patients who test HBeAg negative is a common occurrence (Sakugawa et al., 2001). Determination of HBV DNA also circumvents the problem of demonstrating and staging the infections sustained by genetic variants of HBV, such as the HBeAg-minus mutants (Hatzakis et al., 2006).

HBV DNA can now not only be detected with extremely high sensitivity but also be quantitated with good reproducibility over a wide range of values. The latest generation of quantitative real-time PCR assays can detect as few as 20 HBV DNA copies/ml of plasma or serum with a linear range of 10^9 copies (Hui et al., 2006). To allow meaningful comparison of the results and in response to the need for standardization of quantification, the World Health Organization (WHO) has introduced an international standard based on a high-titer reference HBV isolate of genotype A. One international unit is equivalent to 5.4 copies of HBV DNA, although the conversion factor may vary depending on assay chemistry and procedure (Saldanha et al., 2001). As can be inferred from what was discussed in the prevention and treatment section, quantification of HBV DNA has become a key parameter in the current guidelines for the management of chronic hepatitis B (Ghany and Doo, 2006; Keeffe et al., 2006). In particular, measurement of HBV DNA load in plasma or serum is recommended for the initial evaluation of chronic hepatitis B, for recognizing those patients who are at risk of disease progression and might benefit from treatment, and in the follow-up of responses to therapies. Thus, recently it has been proposed that a level of 20,000 IU/ml (10^5 genomes/ml) can be used as a sort of cutoff to distinguish

TABLE 5 Interpretation of serological and molecular test results for HBV infection

Results[a]							Interpretation
HBsAg	HBeAg	HBV DNA	Anti-HBe	Anti-HBc IgM	Anti-HBc IgG	Anti-HBs	
+	+	+	–	–	–	–	Acute infection, very early stages
+	+	+	–	+	–	–	Acute infection, early stages
+	+	+	–	–	+	–	Acute infection, late stages
–	–	–[b]	+	±	+	+	Resolution of acute infection
+	±	+	–	–	+	–	Chronic infection, typical profile
+	–	±	+	–	+	–	Resolution of chronic infection underway or infection with HBeAg-minus mutant
–	–	+	±	–	±	±	Occult infection
–	–	–	±	–	±	±	Past infection
–	–	–	–	–	–	+	Past vaccination
–	–	–	–	–	+	–	False positive; passively acquired antibody; resolved infection with loss of detectable anti-HBs
–	–	–	–	–	–	–	No present or past infection

[a]+, positive; –, negative; ±, may be either positive or negative.
[b]With the most sensitive detection methods, low copy numbers of HBV DNA may remain demonstrable in the blood of some patients who have resolved an acute infection for protracted periods.

between inactive carrier state and chronic hepatitis and to initiate therapy in the HBeAg-positive patients with moderate-to-severe disease (ALT persistently elevated for 3 to 6 months) and/or with histological demonstration of moderate-to-severe hepatitis. HBeAg-negative chronic patients with compensated cirrhosis have lower median viral loads, and for these subjects, the threshold has been set at 2,000 IU/ml (Ghany and Doo, 2006; Keeffe et al., 2006). The threshold HBV DNA level at which therapy should be initiated, however, is not absolute because viral loads do not necessarily reflect disease severity.

Recently, methods also have been developed for measuring the levels of HBV cccDNA in liver and blood cells. This replicative intermediate becomes the dominant form of HBV DNA during suppression of viral replication and is possibly responsible for viral rebound after short-term antiviral therapy. However, until their utility in patient management is demonstrated, the assays that assess cccDNA should be regarded as research tools (Wong et al., 2006; Bourne et al., 2007).

Assays for Determining HBV Genotype and Drug Resistance

Validated methods for HBV genotype determination include sequencing, genotype-specific PCR, and restriction fragment length polymorphism (RFLP) or hybridization analysis of amplified segments of the viral genome (Bartholomeusz and Schaefer, 2004). So far, the practical utility of these methods has been limited to epidemiological investigations, but with increasing knowledge of the disease potential of the different genotypes, they might become more widely used. Indeed, reports are beginning to appear that describe tentative correlations between HBV genotype, and even subgenotype, and infection outcome, including the development of HBeAg-minus variants (Devesa and Pujol, 2007; Schaefer, 2007). For example, Caucasians have been reported to have a better chance to recover and clear the virus if infected with genotype A than if infected with genotype D, and Asians to develop milder hepatitis and respond better to antivirals if infected with genotype B than if infected with genotype C (Yuen et al., 2003). Sequencing the entire viral genome is the gold standard for genotyping HBV as well as other viruses, since it allows the recognition of recombinant genomes as well as of the standard genotypes. This method is, however, technically demanding and time-consuming, and genotyping is routinely based on the sequencing of selected segments of the genome, most commonly within the S gene, and comparing the sequence data to those of specific prototype HBV isolates retrievable from GenBank (Bartholomeusz and Schaefer, 2004). Genotype-specific PCR are commercially available in various formats (Osiowy and Giles, 2003).

With the introduction in therapy of effective HBV polymerase inhibitors, determination of drug resistance has acquired a significant impact in patient management. In particular, the determination should be carried out when the patient's viral load does not fall by at least 1 $\log_{10}$ unit after 1 month of therapy or increases by 1 $\log_{10}$ unit over the nadir in an initial responder. Currently, the method of choice is sequencing of the P gene in those regions known to be involved in mutations, generally comprised between codon 80 and 250, which singly or in combination, determine drug resistance and then analyzing the sequence by programs available online or with other approaches (Yuen et al., 2007). Commercial methods, based on RFLP or combined hybridization (i.e., immobilization by probes on a solid support), sequencing of PCR amplicons, and other approaches await full validation in the field (Olivero et al., 2006).

HCV

Subsequent to the development of accurate diagnostic assays for HBV infection, the existence of an additional form of transfusion-transmitted hepatitis became apparent. Since the diagnosis was one of exclusion, for several years the disease was referred to as non-A, non-B hepatitis, but the causative agent is now known to be HCV in the great majority of cases. Acute HCV infection is often asymptomatic and may remain undiagnosed. When clinical illness is present, it is generally mild. Because acute infection is so benign, the clinical importance of hepatitis C is mainly due to its remarkable ability to persist and to produce chronic and irreversible liver damage. The majority of acutely infected individuals progress to chronic infection with almost no propensity to resolve the infection spontaneously. Chronic hepatitis C tends to be more slowly progressive than chronic hepatitis B, but the long-term consequences are possibly more severe. For this reason and because of the absolute lack of vaccines, hepatitis C is presently the major public health concern associated with hepatitis viruses.

The Virus

Evidence that a specific virus was implicated in the etiology of what was then known as non-A, non-B hepatitis was obtained when, in the late 1970s, it was shown that chimpanzees could be infected with patients' blood. The agent proved hard to characterize and remained elusive until 1989, when its genome was cloned and described in detail. Because HCV shares significant properties with members of the *Flaviviridae*, it is classified within this family in the genus *Hepacivirus*. The major structural and replication features of HCV are summarized in Table 6, while Fig. 4 provides a schematic representation of its positive-sense single-stranded RNA (ssRNA) genome. Further information can be found in Lemon et al., 2007.

An aspect of HCV that needs to be discussed in more detail because of important implications in laboratory diagnosis as well as in clinical practice is the heterogeneity of its genome in different viral isolates. Variability is especially high in the regions encoding the envelope glycoproteins, with maximum variability in a 30-amino-acid stretch (hypervariable region 1 or HVR1) of the envelope glycoprotein E2, believed to represent a key target for protective antiviral immune responses. The noncoding terminal 5′ untranslated region (UTR), the terminal 100 bases of the 3′ UTR, and the region coding for the capsid protein are by contrast the most highly conserved. Based on this variability, phylogenetic analyses have permitted classification of HCV into at least six genotypes, designated 1 through 6, most of which are divided into a series of subtypes, designated by lowercase letters (Simmonds et al., 2005; Murphy et al., 2007). Coinfections by multiple HCV genotypes and/or subtypes are present in some patients, but it is not known whether they represent the result of simultaneous or subsequent exposures to the virus. Intergenotype (2i/6p, 2/5, 2k/1b) and intersubtype recombinants (1a/1b) of HCV, with crossover points in the NS2/NS3 and NS5B regions, respectively, also have been detected in patients mainly from geographical areas characterized by high HCV genetic diversity, such as Russia, Vietnam, and Peru. In fact, since the genotype of patients' HCV is generally inferred from analysis of a single genomic region (see below), the real frequency of recombinants is likely to be underestimated. Accordingly, the rate of recombination was found to be relatively high in experimentally

TABLE 6 Structure and replication of HCV: major features

Structure
Round-shaped, 55- to 65-nm-diameter virion with density ranging between 1.14 and 1.16 g/ml
Icosahedral 30- to 35-nm-diameter capsid, or core, composed of numerous copies of a single protein (p21)
Envelope with short projections composed of two virus-encoded glycoproteins (E1 or gp35 and E2 or gp70) and cell-derived lipids; E2 contains a hypervariable domain (HVR1)
Linear ssRNA (approximately 9.5 kb) with messenger polarity
Most of the genome is a single ORF, encoding a 3,011- to 3,033-amino-acid precursor from which a dozen structural and nonstructural proteins are formed (Fig. 4); an additional ORF overlapping the C region leads to the production of a further protein dispensable for replication and with no known functions, designated F for frameshift or ARF for alternative reading frame
The two extremities of the genome, the 5′ UTR (324 to 341 nt long) and the 3′ UTR, have no coding functions but possess defined secondary structures and exert essential regulatory functions in genome replication and expression
Replication
Binding to the cell surface through E2 triggers virion endocytosis; cell receptors/coreceptors are poorly understood: molecules that have been implicated include the large extracellular loop of the tetraspanin CD81, scavenger receptor class B type 1, receptor for serum low-density lipoprotein, the mannose-binding lectins DC-SIGN and L-SIGN, surface heparan sulfate, and claudin-1
All the intracellular steps occur in the cytoplasm in apparent association with cytoplasmic membranes
The viral ORF is translated into the polyprotein precursor; translation is cap independent and is controlled by an internal ribosome entry site located in the 5′ UTR
Co- and posttranslationally, the polyprotein is cleaved by viral and, possibly, cellular proteases to generate the three major structural proteins and several nonstructural proteins with catalytic (including an RNA-dependent RNA polymerase, two proteases, and an NTPase/helicase) and regulatory functions
NS3–NS5 proteins form a replication complex associated with an intracellular "membranous web" derived from the endoplasmic reticulum
Synthesis of antigenomic minus-strand RNA, which serves as a template for progeny genomes
Virus assembly and envelope acquisition at endoplasmic reticulum membranes; mechanism of release from cells is poorly understood but probably involves the p7 protein which has ion channel activity
No overt cytopathic effects produced

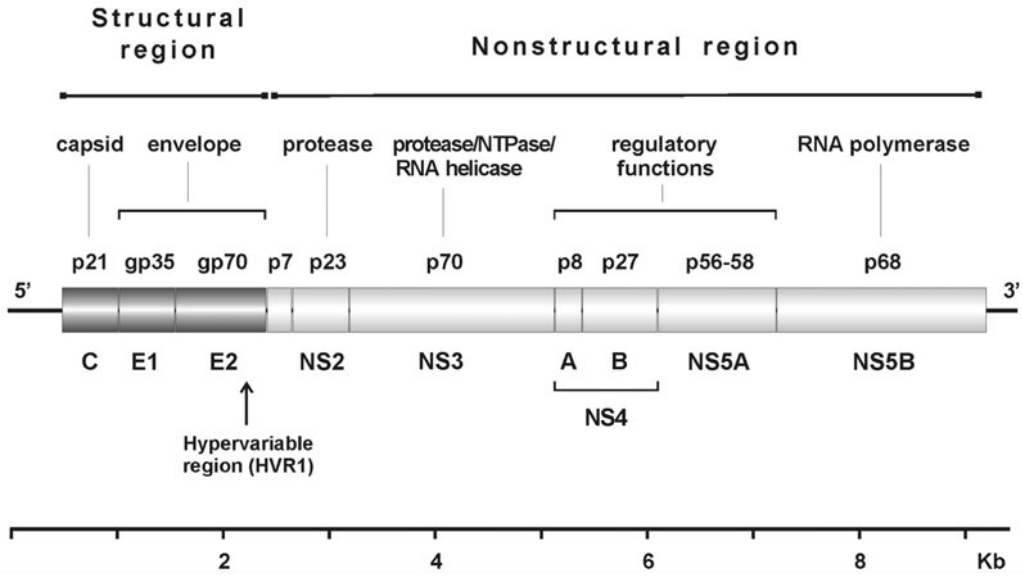

FIGURE 4 Organization of the HCV genome and encoded proteins (Table 6). C, capsid (core); E, envelope; NS, nonstructural. p7 is a small nonstructural protein composed of two transmembrane domains with putative ion channel activity.

infected chimpanzees, which represent the only animal species known to be consistently susceptible to HCV (Gao et al., 2007).

As discussed in more detail below, HCV genotypes display different geographical distributions worldwide, influence the likelihood of a patient's response to therapies, and serve to guide the duration of therapy (see below). Some evidence also has been obtained for the existence of between-genotype differences in disease progression, including observations that genotype 3 is especially associated with liver steatosis (Ramalho, 2003) and that genotype 1 infections have a greater tendency to undergo spontaneous recovery relative to other genotypes (Harris et al., 2007). However, whether or not the different HCV genotypes do indeed present clinically relevant variations in pathogenicity is still a matter of debate. Also essentially unknown is whether the genotypes of HCV have practical importance in terms of cross-protective immunity (Lanford et al., 2004). Observations that hosts infected with a given genotype can be superinfected with another isolate of the same or of different genotype argue against this possibility. Improved in vitro antibody-mediated neutralization assays stemming from recent progress in in vitro propagation of HCV will hopefully shed more light on this aspect.

With regard to virus propagation in vitro, in the past, several primary tissue cultures and established cell lines were reported to support limited growth of wild-type HCV in vitro, but the amounts of virus produced were too low to be of much utility (Lau, 1998; Morrica et al., 1999). More recently, efficient amplification in vitro of infectious HCV has been achieved by transfecting the human hepatoma cell line Huh7 with strain JFH-1 of the 2a genotype (Lindenbach et al., 2005; Lindenbach et al., 2006), with a strain of genotype 1a in which several adaptive mutations were introduced, and with several nonmutated strains belonging to different genotypes (Kato et al., 2007). For a recent review on these aspects, see Sheehy et al., 2007.

Dynamics of HCV Replication in Infected Patients and Antiviral Immune Responses

In infected humans and chimpanzees, HCV shows a distinct tropism for the liver, but there is growing evidence that it also can replicate in other tissues, albeit much less actively. Indeed, although the demonstration of HCV replication in extrahepatic sites is fraught with technical difficulties (Blackard et al., 2006), in situ hybridization, immunohistochemistry, and reverse transcription (RT)-PCR assays specific for antigenomic minus-strand RNA have provided evidence of replication not only in hepatocytes but also in several lymphoid and nonlymphoid tissues, including biliary epithelial cells, bone marrow cells, spleen cells, peripheral blood mononuclear cells (PBMC), circulating plasmocytoid dendritic cells, pancreas, thyroid, and salivary and adrenal glands.

The clinical course and outcomes of HCV infection can be extremely varied and unpredictable. However, since the viral events that characterize the infection are rather stereotyped, the only useful distinction from the clinical virology laboratory standpoint is between acute and chronic infections.

Self-Limited Acute Infections

Information about acute HCV infection (Fig. 5A) is mainly derived from posttransfusion, needle-stick injury, and IDU cases. Under these circumstances, the virus becomes detectable in the bloodstream in 1 to 3 weeks, although it probably reaches the liver much earlier (in chimpanzees, this organ becomes virus positive several days earlier than blood). As discussed below, many forms of hepatitis C occur in patients with no known risk of parenteral infection. Since it is likely that in these cases infection is initiated by small doses of virus gaining access to the body through the mucous membranes, it is also likely that the virus takes longer to become detectable in blood. The extrahepatic sites of virus amplification, if any, in these early stages and the routes the virus uses to reach the liver are unknown. Sixty to 70% of acute infections are completely asymptomatic. When symptoms

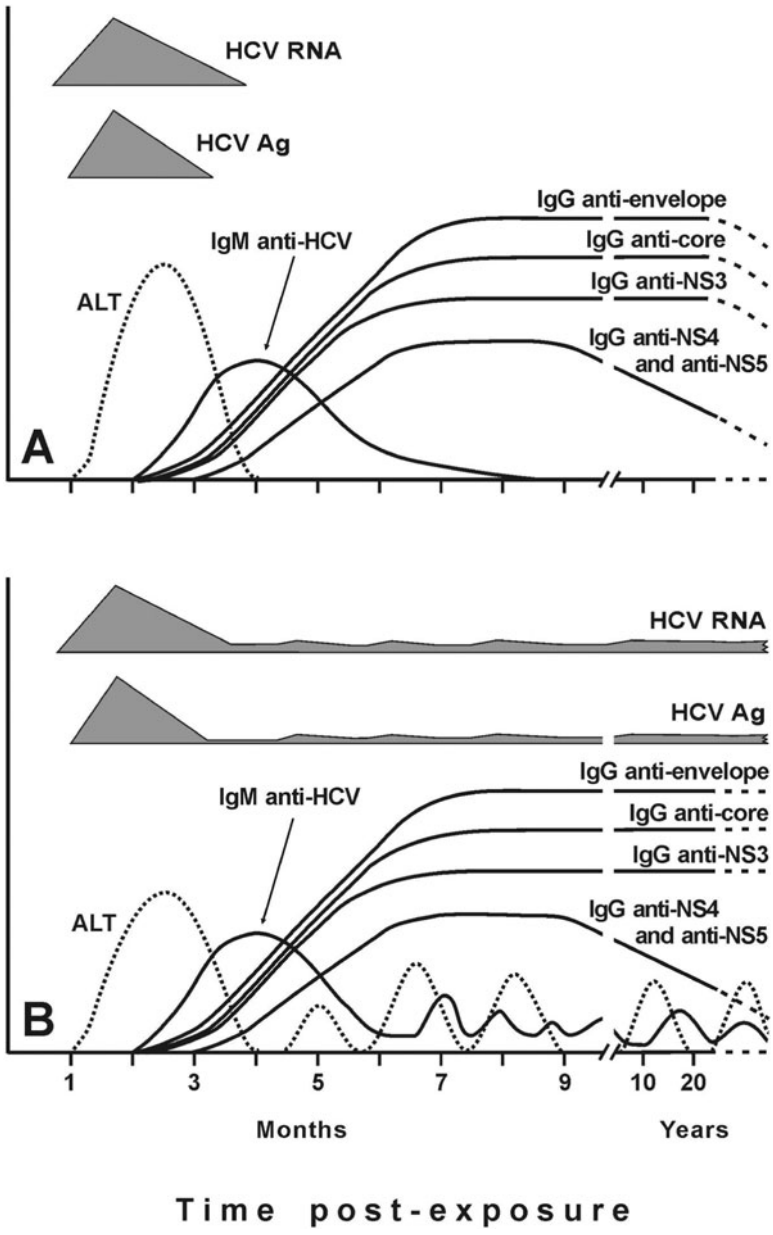

FIGURE 5 Typical courses of HCV infection. (A) Acute, self-limited infection. (B) Infection that does not resolve and becomes chronic. While the virological and immunological events depicted are seen in virtually all patients, clinical symptoms develop in a minority. The shaded areas represent the time periods during which viral RNA and viral antigen are demonstrable in the circulation.

are present, they consist of generally mild jaundice (20 to 30%) or nonspecific manifestations (10 to 20%) after an incubation period of 2 to 26 weeks (average, 7 weeks). ALT elevations may exhibit different patterns (single or multiple peaks, plateaus, etc.) but infrequently exceed 1,000 U/liter, reflecting generally moderate liver damage. Fulminant hepatic failure is rare and usually occurs in conjunction with other hepatotropic noxae.

Plasma viremia peaks at titers of 10^5 to 10^7 IU/ml a few weeks after exposure. In most acutely infected patients, this initial phase of florid virus replication is followed, within 2 to 12 weeks, by a gradual decline of viremia. Symptoms disappear, and ALT concentrations progressively normalize. However, this does not mean that the infection will be cleared. Indeed, complete resolution of primary HCV infection is observed only in 15 to 30% of acutely infected persons in different studies. In these patients, HCV viremia tends to reach lower levels than in patients who will become chronically infected. However, no marker or complex of markers can be used to reliably predict whether acute infection will spontaneously be cleared or become persistent (National Institutes of Health, 2002).

The modulation of viremia described above as well as the outcome of acute infections is believed to be determined by

the response of the host's defenses to the replicating virus. IFN production and other effectors of innate immunity are certainly involved, especially in the earliest stages of infection, although HCV seems to have developed several strategies to limit their antiviral effects (appraisals of our current understanding of how HCV escapes intracellular and immune control can be found in Gale and Foy, 2005, and in Bowen and Walker, 2005, respectively).

On the other hand, adaptive immune responses are vigorous but, for reasons still poorly understood, tend to develop more slowly than in most other systemic viral infections. Thus, CD4- and CD8-mediated responses specific for a variety of viral T epitopes appear in blood 5 to 10 weeks after the initiation of infection, and antibodies do not become demonstrable until 8 to 12 weeks (corresponding to 6 weeks after the onset of viremia, on average), although the preserological phase can occasionally be much longer (up to 9 months in some patients) or shorter (4 weeks). As in most other infections, the first antiviral antibodies detected in infected patients are usually of the IgM class, despite difficulties that still exist for their routine demonstration and measurement (see below). IgG antibodies, which are usually restricted to the IgG1 subclass (Netski et al., 2005), develop later and with kinetics that vary depending on the viral antigen considered: in particular, those to the envelope, core, and NS3 antigens usually develop earlier than antibodies to NS4 and NS5.

Earliness and strength of the adaptive responses are generally believed to dictate strongly the outcome toward control or persistence of HCV. However, many aspects of how this occurs remain to be worked out. While general consensus exists that CD4-mediated responses present a greater breadth and intensity in patients with a self-limited infection than in patients who become chronically infected, the role of CD8-mediated responses in determining the clearance of acute infections has often been questioned (Ferrari, 2007). On the other hand, understanding the role of antibodies in limiting HCV infection has been hindered by the lack of reliable serum neutralization assays. Recently, however, the development of neutralization assays exploiting retroviruses pseudotyped with HCV envelope glycoprotein or HCV strains that have been propagated in cell culture is providing insights. Neutralizing antibodies could be detected in the majority of chronically infected patients but only in a low proportion of the self-limited infections examined (Logvinoff et al., 2004), and in a group of hemodialysis patients with accidental acute infection, the development of neutralizing antibodies was found to correlate temporally with the decrease of initial viremia levels (Lavillette et al., 2005). However, the impact of neutralizing antibodies in the natural history of HCV infection is still controversial, as exemplified by one study in which these antibodies did not correlate with spontaneous resolution of acute infection (Kaplan et al., 2007). Furthermore, a recent study has demonstrated that antibodies obtained from patients at a given time fail to neutralize the concomitant virus although they neutralize virus samples collected at earlier times, thus showing that HCV can continuously evolve to escape antibody neutralization (von Hahn et al., 2007).

It is important to note that HCV-specific cell-mediated immunity may still be detectable many years after recovery from acute infection and that antibody titers slowly decrease and may become undetectable within 10 to 20 years. Also, virus-specific CD4 and CD8 T cells may actually represent the only marker of previous infection detectable in the peripheral blood of anti-HCV and HCV RNA-negative individuals with documented exposure to HCV (Al-Sherbiny et al., 2005; Kubitschke et al., 2007) and in those with a diagnosis of "occult HCV infection," discussed below (Quiroga at al., 2006).

Chronic Infections

As discussed above, the majority of HCV-infected individuals become virus carriers indefinitely. After the acute phase, the persons who have not resolved the infection are usually symptom free, with ALT levels in the normal range or only slightly elevated, for at least 1 or 2 decades (Fig. 5B). However, with further duration of infection, in most of these subjects, ALT levels become intermittently or persistently elevated, and liver function tends to progressively deteriorate as a result of accumulating hepatocellular damage. Others maintain constantly normal ALT values but nevertheless are viremia positive, and a liver biopsy may reveal considerable damage, ranging from persistent or active hepatitis to cirrhosis. Still others do not appear to develop clinically significant histopathological changes. Eventually, cirrhosis develops in 20% of persistently infected individuals, and HCC occurs in up to 2 to 3%. Male gender, old age at the time of infection, ethanol intake, high necroinflammatory activity in the liver, coinfection with HBV and HIV, and specific genetic traits of the host that are currently being recognized (Asselah et al., 2007; Huang et al., 2007) are all factors that negatively affect prognosis.

It is generally accepted that patients with chronic HCV infection, even if they show an indolent course, should be evaluated for severity of hepatic damage and possible treatment and that accurate prognosis requires long-term follow-up with periodic measurement of ALT levels. In the absence of antiviral treatments, viremia levels may vary between 10^3 and 10^7 IU/ml (usual range, 10^5 to 10^6 IU/ml) in different chronic patients but remain relatively stable over time in each individual patient, with variations in titer that usually do not exceed 1 log unit; intermittent viremia is uncommon. Illuminating data on the kinetics of HCV replication during chronic infection have been obtained by sequentially measuring the viremia levels of responder patients given IFN-α at doses sufficient to markedly reduce virus production and release from infected cells. The half-life of virions in plasma was estimated to be less than 3 h, while the number of virions produced and cleared per day was in the order of 10^{12} (Neumann et al., 1998). In chronically infected individuals, spontaneous resolution of infection is a rare although not impossible event (Scott et al., 2006). On the contrary, HCV RNA has been detected in PBMC and the livers of patients who have apparently undergone a well-documented spontaneous or therapy-induced clearance of viremia (Pham et al., 2004; Stapleton et al., 2004; Carreno et al., 2006). These findings have led to the definition of a new form of infection, labeled "occult HCV infection," characterized by abnormal liver function tests and presence of both genomic and antigenomic HCV RNA in the liver and often also in the PBMC, with no HCV RNA nor antibodies detectable in serum (Carreno, 2006). Recent data obtained by ultracentrifuging serum prior to RT-PCR analysis suggest that failure to detect HCV RNA in the serum of these patients is most likely due to the fact that their viral loads are too low to be detected even using the most sensitive techniques currently available (Bartolomè et al., 2007). Clinical and epidemiological implications of this novel form of infection remain to be clarified.

The spectrum of antiviral antibodies found in chronically infected patients is approximately the same as that observed

in advanced acute infections, even though antiviral IgM tends to be detectable in fewer patients and most often in a discontinuous fashion, and anti-NS4 and anti-NS5 antibodies may slowly decline considerably and occasionally even become undetectable after years of persistence. CTL and other effectors of cell-mediated immunity specific for HCV epitopes are also readily detected in the peripheral blood and intrahepatic lymphocytes of most chronically infected individuals. However, attempts to correlate these immune parameters with how the infection evolves and with the severity of liver damage have so far been disappointing. An important contribution of cell-mediated immunity to both containment of infection and generation of hepatocellular injury is, however, considered likely (Bowen and Walker, 2005; Spengler and Nattermann, 2007).

Epidemiology

The WHO estimates that HCV infection affects about 180 million people across the globe, some 3% of the world's population, and that 3 to 4 million persons are newly infected each year. Prevalence is low (<1%) in Northern Europe, Canada, and Australia, about 1% in the United States and most of Europe, and high (>2%) in many countries in Africa, Latin America, and Central and Southeastern Asia, where prevalence rates between 5% and 10% or more are common. In particular, a recent survey of representative households has estimated that 4.1 million people are anti-HCV positive in the United States, excluding incarcerated and homeless persons (Armstrong et al., 2006). Also, an estimated 8,000 to 10,000 HCV-related deaths occur in the United States each year.

Infected humans are the only source of HCV contagion. In developed countries, the highest prevalence is found in males in their 40s and 50s belonging to low socioeconomic classes, and the primary mode of transmission is currently the sharing of injection equipment among IDUs (Shepard et al., 2005). Prevalence rates in IDUs range between 50 and 90% (Armstrong et al., 2006). However, HCV diffusion is significantly higher in all the categories at risk for parenteral infection than in the general population. For example, it can be as high as 60% in hemophiliacs who received commercial clotting factors before these blood derivatives were treated to inactivate viruses. Posttransfusion infections have dropped dramatically since proper screening of donors has been introduced. Where accurate procedures for the safety of donations are implemented, the risk is now calculated to be on the order of 1 per 2 million units transfused. However, in resource-poor countries, blood transfusions still represent an important mode of HCV spread (Shepard et al., 2005). Prior to the implementation of virucidal treatments, intravenous immune globulins could also transmit the infection. Renal patients requiring dialysis are also frequently infected. Individuals occupationally exposed to blood are also at increased risk of infection, and there are even sporadic reports of HCV transmission from infected health care personnel to patients. Needle stick accidents and other types of percutaneous or permucous exposure represent important modes of transmission. Of note, iatrogenic transmission is believed to have contributed substantially to HCV diffusion in Egypt and possibly other countries.

The HCV infections that occur in people with no identifiable parenteral risk are called sporadic or community acquired. Understanding the sources and modes of contagion in these people is difficult, in part probably due to hesitance to disclose previous risk behaviors. There is evidence that HCV may be shed with genital secretions and saliva. Both mother-to-child and venereal transmissions are known to occur but are considerably less efficient than for HBV, unless expecting mothers and sexual partners have especially high viremia levels due to acute infection, concomitant HIV infection, or other reasons. Approximately 5 to 10% of infants born to infected mothers are virus positive in different populations. Transmission is believed to occur in utero, as a consequence of a high viral load in the mother. Spread to household contacts is infrequent. There are also no indications that HCV or other blood-borne hepatitis viruses can be spread by insect vectors.

Investigations have shown that genotypes 1 to 3 and their subtypes are ubiquitous, but their relative prevalence rates vary in different geographical regions. Subtype 1a is predominant in North America, and subtype 1b is predominant in Western Europe and Japan. Genotypes 4 to 6 appear to be less common and restricted to specific regions (types 4 and 5 to Africa and type 6 to Southeast Asia), although a map of HCV genotype distribution is still incomplete. Subtype 2c, previously considered a rare genotype, was found to represent the second most frequent genotype in Italian patients with community-acquired infection when an assay that identified this subtype was used (Maggi et al., 1997b). Of note, genotype distributions in Europe have been found to vary with patients' age, probably reflecting temporally distinct, independent epidemic waves. Thus, subtype 1b has been found to be responsible for over 80% of infections in persons 60 years old or more but for fewer than 30% of the cases in people less than 30 years old. Differences in genotype distribution have also been observed, depending on mode of infection. Thus, hemophiliacs tend to be infected with genotypes that are predominant in the areas from which the contaminated blood used for clotting factor production derived, rather than with those most prevalent in their own countries (Kinoshita et al., 1993; Pistello et al., 1994).

Prevention and Treatment

Except for the lack of an efficient vaccine, primary prevention of HCV infection relies on measures similar to those discussed for HBV. Because many infected people may be unaware of their infections, identification of HCV-positive persons for appropriate counseling and management and routine testing for those at increased risk are highly recommended public health measures. Interrupting virus transmission through iatrogenic or accidental exposure to infected blood is also of primary importance, as exemplified by the greatly reduced number of infections currently observed posttransfusion and posttransplant. Blood derivatives are now also safe due to improved blood supply and virucidal treatments. In the absence of protective proteins, HCV is unstable at room temperature; however, complete inactivation of infected plasma requires heating it to 100°C for 5 min. Infectivity is readily destroyed by ether, chloroform, β-propriolactone, and formalin. Secondary prevention practices are similar to those outlined for HBV-infected patients.

IDUs recovered from a previous acute HCV infection appear to have a reduced risk of developing viremia after reinfection compared to infection naive IDUs (Grebely et al., 2006), and similar findings have been reported in experimentally infected chimpanzees. However, the efficacy of postexposure prophylaxis with immune globulins is considered marginal or none. The development of vaccines is progressing, albeit slowly. Immunization of experimental animals with different recombinant viral proteins and polyproteins or genetic vaccines has prevented acute infection or reduced the rate of progression to chronic infection after challenge,

but the immune correlates of protection are still elusive. Vaccines based on recombinant E1 and E2 glycoproteins are currently in phase 1 clinical trial in humans (Houghton and Abrignani, 2005; Vajdy et al., 2006).

The current standard of care for acute and chronic infection is pegIFN-α in combination with ribavirin, which leads to permanent clearance of the virus in approximately 60% of patients. The reason this combination therapy is active is largely unknown. IFN is believed to act by inhibiting viral replication and stimulating antiviral immune responses, while suggested mechanisms for ribavirin activity include modulation of the Th1-Th2 balance, inhibition of the NS5B polymerase, depletion of intracellular guanosine triphosphate pools, accelerated clearance of infected cells, and more recently, an accumulation of mutations in the viral genome resulting in impaired replication fitness (Feld and Hoofnagle, 2005; Pawlotsky, 2005).

It is generally agreed that chronic hepatitis C patients with persistently elevated ALT, high viremia levels, and liver biopsy specimens showing portal and bridging fibrosis and significant necroinflammatory changes should be treated because their risk of developing cirrhosis is greater. There is also wide consensus that patients with persistently normal ALT and those with advanced cirrhosis should be managed on an individual basis or in the context of clinical trials. The response rate to combined therapy in patients with normal serum liver enzymes is similar to that of individuals with elevated enzymes (Strader et al., 2004; Zeuzem et al., 2006). The best modalities for treatment of acute infections are less well defined; however, prospective randomized trials have established that antiviral therapy should start as early as possible after presentation (Kamal et al., 2006).

The goal of therapies is an SVR, defined as HCV undetectable in blood 24 weeks after the end of treatment. When this is achieved, long-term follow-ups demonstrate that the response is durable in >95% patients. Standard combination therapy produces, in the majority of responders, a biphasic decrease of viral load, namely a rapid initial drop of 1 to 2 $\log_{10}$ units after 24 or 48 h and a slower second phase of viral decay over the subsequent weeks. In genotype 2- or 3-infected patients, the size of the early drop can be as high as 3 or 4 $\log_{10}$ units, whereas in some patients, especially those infected with genotype 1, the first phase can be followed by an intermediate one, of approximately 7 to 28 days, during which viremia remains unchanged or increases slightly. Nonresponders typically lack the first phase (null responders) or the second phase (flat responders) (Perelson et al., 2005; Herrmann and Zeuzem, 2006).

The therapeutic problems posed by nonresponder and relapsing patients are the subject of intensive clinical investigation and debate. Strategies considered include more drastic IFN-α regimens, different types of IFN (consensus pegIFN-α, pegIFN-β, IFN-α fused to human serum albumin), ribavirin analogues or prodrugs, and alternative combination therapies. On the other hand, improved knowledge of the potential viral target is guiding the design of new drugs capable of inhibiting viral enzymes (NS3/4A serine-protease, NS5 RNA-dependent RNA polymerase, and helicase) or blocking cell entry. Other therapeutic approaches being explored include antisense or interfering RNAs, ribozymes, agonists for Toll-like receptors, and immunomodulators (De Francesco and Migliaccio, 2005; He et al., 2007).

Currently, HCV-associated cirrhosis and HCC are the indication for approximately half of the cases of liver transplantation. Following transplantation, however, HCV-induced graft hepatitis and fibrosis/cirrhosis occur in up to 90% and 10% to 30% of recipients, respectively. Disease progression is accelerated and associated with a higher mortality rate than hepatitis in nontransplanted patients (Brown, 2005). Viral factors believed to accelerate allograft fibrosis include high pretransplant and/or early posttransplant viral load, infection by genotype 1b, and high genetic complexity of the viral quasispecies (Berenguer, 2005).

Indicators Predictive of Beneficial Response to Antiviral Treatments

The limited efficacy of HCV therapies must be weighed against their considerable side effects and cost. Therefore, criteria that predict the likelihood of an SVR have been and still are actively sought. Data indicate that several variables influence the outcome of treatment, but none is 100% predictive. Old age, male gender, African-American ethnicity (Conjeevaram et al., 2006), high body weight or body mass index, ethanol or intravenous drug use, advanced hepatic fibrosis and cirrhosis, coinfection with HIV, failure to respond to previous anti-HCV treatments, high viremia titers, and elevated liver enzyme concentrations in blood are considered dependable predictors of poor responsiveness. The viral genotype is also an important determinant, since the rates of response are higher against genotypes 2 and 3 than against genotypes 1 (especially subtype 1b) and 4. Standard pegIFN-α plus ribavirin therapy leads to an SVR in approximately half of the genotype 1- or 4-infected patients after 48 weeks of treatment and in approximately two-thirds of patients harboring genotype 2 or 3 treated for 24 weeks, who also can be given reduced doses of ribavirin (Strader et al., 2004). Information on the therapy requirements of patients infected with genotypes 5 and 6 is limited; however the former are likely to respond similarly to genotype 2- or 3-infected patients, while the latter are believed to present an intermediate responsiveness to therapy (Nguyen and Keeffe, 2005). Comparative genome analysis of genotype 1b isolates obtained prior to IFN treatment from responder and nonresponder Asian patients also has suggested that responsiveness correlates with the amino acid composition of a small segment of the viral protein NS5A, named IFN sensitivity-determining region (Enomoto et al., 1996), but controversial findings have been reported in other parts of the world (Pascu et al., 2004; Brillett et al., 2007; Wohnsland et al., 2007). Additional domains of this protein also have been proposed as determinants of responsiveness, but the data are still inconclusive (Layden-Almer et al., 2005). An elevated quasispecies complexity of HCV also has generally, albeit not invariably, correlated with poor responses and high relapse rates (Chambers et al., 2005; Salmeron et al., 2006).

Considerable information also has accumulated on the predictive value of the viremia changes brought about by the early doses of treatment (Layden-Almer et al., 2006). A 2 $\log_{10}$ unit drop at week 12 (early virologic response) is considered typical of patients with a high likelihood of achieving an SRV at the end of treatment. In its absence, current guidelines state that therapy may be discontinued, especially in genotype 1-infected patients (Strader et al., 2004). Attempts are also being made to establish even earlier predictors of nonresponse. After 4 weeks of treatment, nearly 90% of patients with undetectable HCV RNA in plasma (rapid virological response) achieved an SVR at 12 weeks compared to approximately 50% among those who were still viremia positive (Ferenci et al., 2006; Jensen et al., 2006). Recently, patients coinfected with HCV genotype 3 and HIV-1 with HCV RNA levels below 600 IU/ml at 4 weeks

of therapy were found to have a reduced risk of relapse after 24 weeks (Crespo et al., 2007).

Laboratory Diagnosis and Follow-Up

The first demonstration that an individual is HCV infected is generally obtained by examining serum or plasma with a combination of immunological and molecular assays (Fig. 6). Instead, clinical management of infected patients relies almost exclusively on molecular methods that quantitatively assess HCV replicative activity.

Tests for HCV Antibodies

In acute HCV infections, the appearance of viremia precedes that of biochemical indicators of liver damage by 3 to 10 weeks and that of antiviral antibodies by several more weeks. Thus, in the early phase when antiviral antibodies are not manifest (the window period), infection can be demonstrated solely by testing for viremia. However, the laboratory is rarely requested to perform a diagnosis of HCV infection this early in infection. In most instances, the patient under scrutiny is being evaluated for symptoms suggestive of ongoing hepatitis or, more often, has already been found to have altered ALT levels during routine blood testing performed for unrelated reasons. Because by the time ALT levels are elevated most patients possess detectable antiviral antibodies, the first approach to the diagnosis of HCV infection is usually via serology. It should be noted, however, that roughly 10% of immunosuppressed patients, including those in long-term dialysis, may test antibody negative.

The serological approach to diagnosis of HCV infection is now generally done through the use of commercial assays that have been redesigned several times to shorten the duration of the preserological window period (which now ranges between 4 and 6 weeks) and increase sensitivity and specificity, thus meeting the needs of blood banks as well as of clinical virology laboratories. These kits consist of various formats of EIA and chemiluminescence immunoassays (CIA) prepared with carefully selected recombinant and synthetic antigens derived from conserved domains of the core, NS3, NS4, and NS5 regions of the viral genome. Their specificity is generally excellent, and false-positive results are very limited (due to hypergammaglobulinemia, aged sera, rheumatoid factors, and unexplained causes). Nonetheless, especially when persons with normal ALT levels are being tested, it is correct to verify positive EIA or CIA results with supplemental serological tests, such as immunoblot assays that permit dissection of the specificity of reacting antibodies. With the latter assays, sera are considered positive when they are reactive with epitopes derived from at least two viral regions and indeterminate when they react with a single region. Particularly in the individuals at low risk for HCV infection, indeterminate immunoblot results may be indicative of false positivity. However, it is important to keep in mind that, since anti-NS4 and -NS5 antibodies tend to be produced later than other antiviral antibodies and may wane following spontaneous or posttreatment resolution of infection, the absence of these reactivities may be indicative of a recent infection still in the process of seroconversion or of a resolved

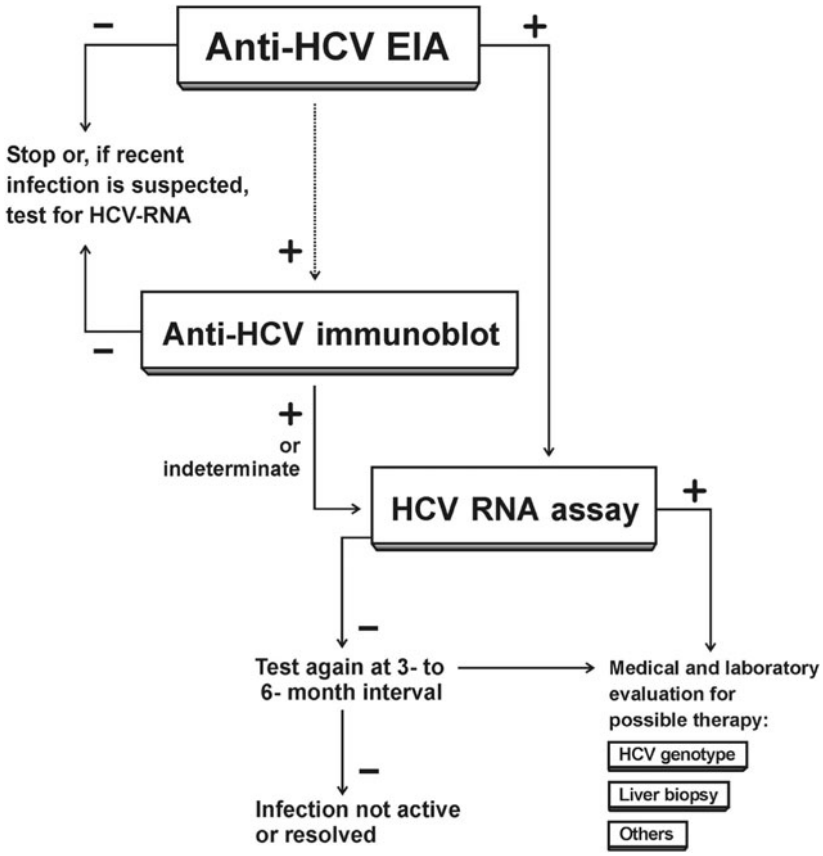

FIGURE 6 Algorithm for the laboratory diagnosis of HCV infection. Note that, although less sensitive, HCV antigen testing can substitute for HCV RNA testing when this is not available.

infection. Indeterminate immunoblot results are also frequently observed in immunocompromised patients whose reactivity is often restricted to core and NS3 antigens. For patients at high risk of infection with increased ALT levels and high anti-HCV EIA or CIA reactivity, supplemental serological testing can be skipped, since correlation between a positive result and infection is essentially 100%, and it is cheaper to proceed directly to testing for viremia.

Even with the optimized assays presently in use, no consistent correlation has been observed between serological parameters and the stage or other variables of HCV infection and disease. Even the presence of virus-specific IgM is poorly informative, since this antibody is detected in 50 to 90% of acute infections versus as many as 50 to 70% of chronic infections. Thus, although in some studies IgM levels have been seen to correlate with ALT or viremia levels and the presence of IgM has been suggested to be predictive of a poor responsiveness to therapy, it is unlikely that the anti-HCV profile will one day become truly useful for staging HCV infection and identifying periods of particularly intense viral activity. Furthermore, the sensitivity and specificity performance of presently available commercial assays for anti-HCV IgM (generally based on viral core antigens, although other antigens also have been used) is not completely satisfactory. Since the avidity of anti-HCV IgG has been shown to increase significantly with the duration of infection, tests that measure this parameter also have been proposed but have not become routine.

Tests for the Detection and Measurement of HCV RNA in Plasma

The assays for anti-HCV have the limits discussed above, and there are no cell culture systems that can be used for demonstrating the presence of HCV in clinical specimens. It is, therefore, not surprising that the molecular assays that detect and quantitate the copies of viral genomes in plasma or serum have come to dominate the diagnosis and management of HCV infection. With the current assay, HCV RNA becomes detectable in blood within 1 to 3 weeks postexposure. During this early phase, the numbers of viral genomes found in the blood of acutely infected patients can exceed 10^7 IU per ml, while they usually range around 10^5 to 10^6 IU/ml in untreated chronically infected patients. In the context of first diagnosis of HCV infection, testing blood for viral RNA is particularly useful for identifying patients who are still in the preserological phase, resolving indeterminate serological results, recognizing those patients who possess anti-HCV as a result of a spontaneously resolved infection, and discriminating between the newborns from HCV-infected mothers who have become vertically infected and those who have merely received transplacental anti-HCV. HCV RNA detection methods can be quantitative or purely qualitative. The latter are especially useful for establishing the safety of blood and organ donations. In patients already known to be infected, HCV RNA quantitation, together with measurement of ALT levels, is the best noninvasive approach for monitoring how the infection is evolving, deciding whether and when to start treatment, and assessing how patients are responding to antivirals. In contrast, while a correlation between severity of liver disease and extent of viral replication in the liver, evaluated as the percentage of hepatocytes harboring replicative-intermediate HCV RNAs, has recently been demonstrated in genotype 1-infected subjects (Pal et al., 2006), it is generally recognized that the size of plasma viral load is of limited relevance for prognosis (Chemello et al., 1994). Indeed, a positive correlation between HCV RNA levels in plasma and viral replication in the liver has been observed only in few studies.

The technologies used by the current commercial assays for HCV RNA detection and measurement include target amplification methods (RT-PCR), transcription-mediated amplification, and signal amplification methods, the specificity, sensitivity, and hands-on requirements of which have been continuously improved. Lately, real-time RT-PCR assays have become increasingly used, thanks to their wide dynamic range, high speed, and simplicity as well as reduced risk of contamination. Currently, the lower limits of detection of commercial kits are very low, ranging between 5 and 50 IU/ml, and intra- and interassay variability have been markedly reduced. However, it is advisable to repeat the tests several times to minimize false-negative results and identify patients who might have intermittent viremia, as is often seen during the course of spontaneous resolution or therapy. Also, although the introduction of IU to express viral loads has greatly facilitated the comparison of results obtained with different methods, it is still recommended that each patient be constantly monitored by using the same type of assay (Caliendo et al., 2006; Sarrazin et al., 2006, Michelin et al., 2007).

Due to the high genetic variability of HCV, correct selection of the viral genome segment targeted is critical for satisfactory performance of gene amplification assays. Despite being the most conserved region of the genome, even the 5′ UTR presents sufficient sequence diversity between genotypes to affect assay sensitivity. In addition, sequence differences outside it can influence 5′ UTR tertiary and quaternary structure and affect 5′ UTR unfolding. These circumstances explain why the first versions of commercial kits for HCV RNA measurement underestimated the titers of genotypes 2 and 3 by approximately 1 $\log_{10}$ unit. The versions currently marketed have been remodeled to reduce this shortcoming, although they probably will need further balancing, especially to achieve a reliable assessment of genotypes 4, 5, and 6 (Caliendo et al., 2006; Gelderblom et al., 2006). Finally, the possibility of incorrect determinations demands that rigorous quality assurance controls be in place in the performing laboratory. The availability of automated devices for nucleic acid extraction, detection, and quantitation has markedly reduced the risk of specimen contamination, and incorrect determinations are usually the result of carelessness in the preanalytical phase.

Tests for the Detection of the HCV Core Antigen in Blood

The immunoassays that demonstrate and quantitate the C antigen of HCV in serum were developed after the genome-targeted molecular methods had already become widely used. Generally, these assays use EIA technologies and include an immune complex dissociation step to allow detection of the antigen, both bound and unbound to antibody. Their use has been proposed as a relatively cost-effective and easy to perform alternative to the use of molecular assays for HCV RNA detection in the diagnosis of acute HCV infection, in assessing chronic infections, and in monitoring responses to therapy (Veillon et al., 2003). Although the tests for C antigen detection show high specificity and perform equally well regardless of the infecting HCV genotype, they are rather insensitive when the viral load is <20,000 IU/ml (the lower limit of sensitivity is on the order of 1.5 pg, corresponding to approximately 12,000 IU of HCV RNA) and become positive a few days after HCV RNA detection tests. Assays that simultaneously reveal C antigen and antibodies also have

been developed, but they have a reduced sensitivity (Laperche et al., 2005). Overall, the tests for C antigen or combined antigen-antibody detection appear to have appreciable utility only when molecular testing is unavailable.

Assays for Determining HCV Genotype

Numerous studies have retrospectively or prospectively investigated the existence of possible associations between specific HCV genotypes and clinical and virological features of the infections produced, but in general, the conclusions have been negative or controversial (Simmonds, 2004). Thus, presently the only widely accepted correlation is that between the patient's genotype and responsiveness to antivirals (see above). However, since this is a critical issue, the laboratory is often requested to perform this classification.

Sequence analysis of the viral genome is considered the gold standard for genotyping HCV, and it also has been shown that sequencing selected segments of the E1, C, or NS5B regions suffices for correct genotype and subtype recognition of a viral isolate (Simmonds et al., 2005; Murphy et al., 2007). However, the recent identification of recombinant forms of the virus indicates the necessity to analyze more than one region when accurate typing needs to be performed for molecular epidemiological studies. For practical purposes, most significantly for predicting the likelihood of a positive response to therapy, sequencing the 5′ UTR, albeit inefficient at classifying subtypes (Bukh et al., 1995; Pistello et al., 1999; Hraber et al., 2006), is considered acceptably accurate, especially in areas where HCV is characterized by a low genetic diversity, such as North America and Europe. In contrast, in areas where genotype 6 HCV is common, such as Southeast Asia, other subgenomic regions, notably within NS5B, which is known to be especially informative for genotyping purposes (Murphy et al., 2007), need to be analyzed to permit genotype 6 differentiation from genotype 1.

Commercial kits for semiautomated sequencing of the 5′ UTR are available, and at least one prototype NS5B-based kit is currently under evaluation. Nevertheless, HCV genotyping by sequencing remains cumbersome, time-consuming, and expensive. Alternative, easier-to-perform methods that have been proposed include type- or subtype-specific RT-PCR, RFLP analysis of selected RT-PCR amplicons, and others (Weck, 2005). A widely used commercial test exploits the ability of selected HCV amplicons to hybridize to type-specific probes immobilized on nitrocellulose strips (line probe assay). A recently updated version of this assay uses primers and probes based on the C region in addition to those derived from the 5′ UTR (Noppornpanth et al., 2006). The manufacturer claims that this version correctly identifies genotype 1 subtypes and distinguishes genotype 1 from 6 in areas where these are highly prevalent, but subtyping of genotypes 2 and 4 has not been improved (Bouchardeau et al., 2007). Recently, two additional methods have become available as analyte-specific reagents. One uses a real-time multiplex technique with primers and probes for the 5′ UTR and NS5B regions and identifies the major genotypes and subtypes 1a, 1b, 2a, and 2b as well (Cook et al., 2006). The other differentiates genotypes 1 through 6 using cleavase and the fluorescent resonance energy transfer technology with genotype-specific probes derived from the 5′ UTR but does not identify subtypes (Germer et al., 2006). The results so far reported suggest that these assays can diagnose mixed infections that are not confirmed by clonal analysis and may fail to type genotypes 1 to 3 when viremia is low (<300 IU/ml). How correctly these assays perform at typing other genotypes remains to be evaluated.

Information about the patient's genotype also can be obtained by a completely different approach which has improperly been defined "serotyping," in that it exploits the fine serological specificity of the anti-HCV developed. The antigens used are genotype-specific synthetic peptides deduced from the C and NS4 regions of the viral genome, and therefore, the method makes no attempt to distinguish subtypes. However, the approach has the benefit of detecting more multiple genotype infections than molecular assays. In essence, all "rapid" genotyping methods have advantages and disadvantages in terms of sensitivity, specificity, and hands-on time (Weck, 2005), which need to be evaluated in the context of the genotypes prevalent in the area where they are used (Vatteroni et al., 1997).

Assays for Analyzing the Quasispecies of HCV

Similar to many other RNA viruses, HCV is present in infected hosts in the form of a variably complex mix of genetic variants, generally known as quasispecies. Since the quasispecies nature of viruses has been invoked to explain many aspects of their natural history (Maggi et al., 1997a), it has been suggested that the quasispecies characteristics of HCV contribute to the determination of clinically relevant features of the infections produced. In particular, these features have been considered a possible further element upon which to base a prediction of responsiveness to treatments. However, knowing the composition of the viral quasispecies has added little or nothing to the predictive value of knowing viral genotype and load (Chambers et al., 2005; Farci et al., 2000; Salmeron et al., 2006). Thus, quasispecies analysis has remained a research tool. Also, the methods used (cloning, sequencing, and single-strand polymorphism analysis, etc.) are cumbersome and need to be performed in laboratories with highly skilled personnel.

HDV (DELTA VIRUS)

HDV, also known as delta virus, is a defective viroid-like satellite virus that replicates productively only in cells infected with HBV and, for this reason, is found only in people infected with this virus. Especially when superimposed on a preexisting HBV infection, acute HDV infections are often associated with an appreciable aggravation of the underlying liver disease and with the highest rate of fulminant courses among the hepatotropic viruses; in contrast, the manifestations associated with chronic HDV infections may either not substantially differ from those caused by HBV alone or, frequently, lead to a more rapid progression to liver cirrhosis compared to single infections with HBV or HCV. Furthermore, the incidence of HCC in patients doubly infected with HBV and HDV is higher than in HBV monoinfection.

The Virus

HDV was first demonstrated in the late 1970s, when a nuclear antigen (delta or HD antigen [HDAg]) that had previously been identified in the hepatocytes of a subset of HBV-infected patients was recognized to belong to a separate, albeit HBV-dependent, viral entity. Cloned and sequenced in 1986, the virus has a number of interesting and unusual properties (Fig. 7 and Table 7) that make it unique among known animal viruses, including a remarkable structural, but not functional and phylogenetic, similarity of its tiny negative-sense ssRNA genome with those of viroids and analogous infectious RNA agents of plants. The gene products HBV supplies to HDV are essential structural components, namely, the envelope glycoproteins representing HBsAg,

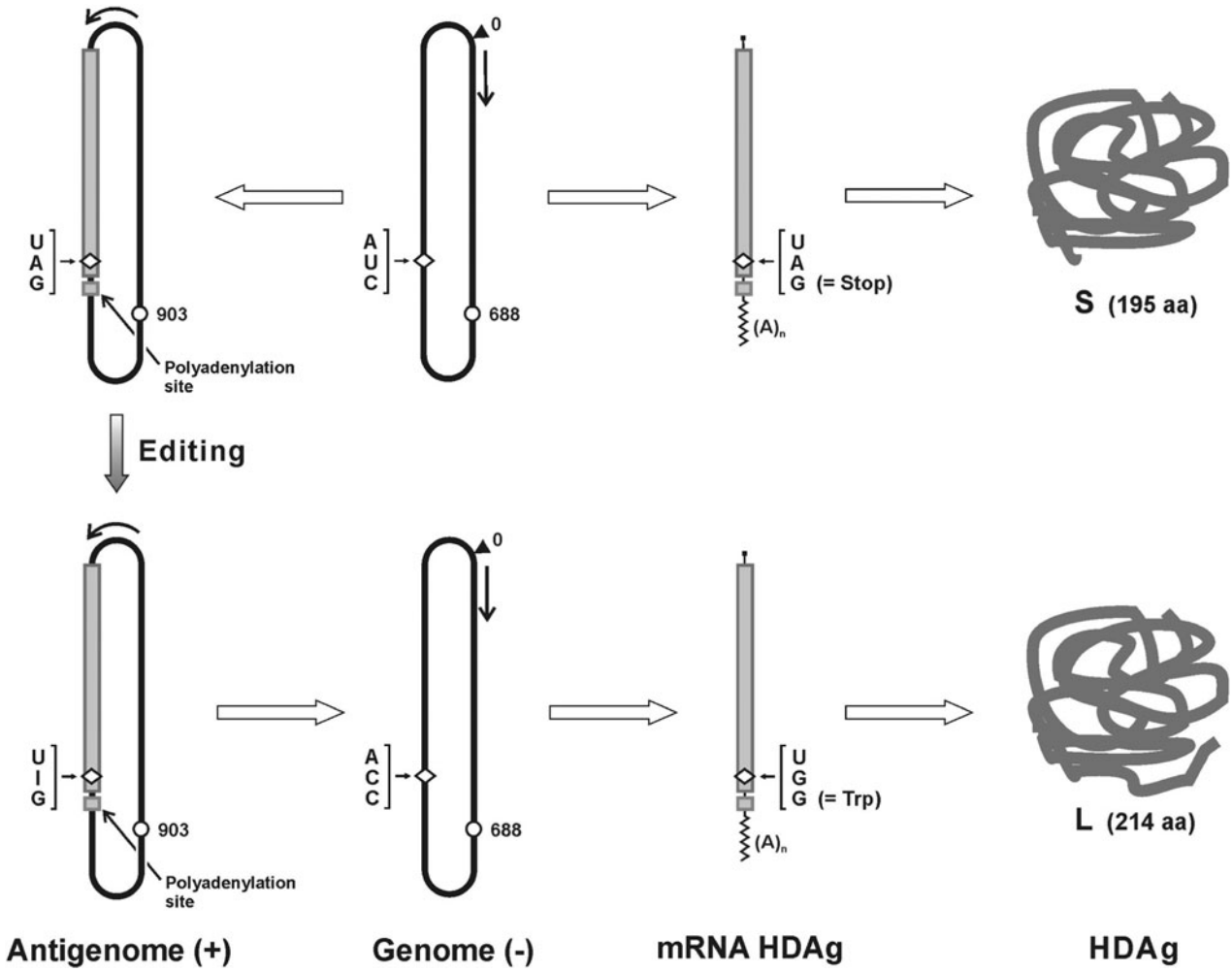

FIGURE 7 Organization of the HDV genome and encoded proteins. The ellipses represent the genomic and antigenomic circular viral RNA. The shaded segment of the antigenome represents the HDAg gene. A variable proportion of antigenomes is modified by host cell enzymes at a specific nucleotide position, the editing site (diamond), contained in the functional ORF. This editing leads to the substitution, in the HDAg mRNA, of an amber stop codon with a tryptophan codon (W) and results in the readthrough of 19 additional amino acids. By this mechanism, variable proportions of S-HDAg and L-HDAg are produced. The open circle indicates the cleavage site in which the viral RNA autocatalytically cuts and ligates itself (Table 7). The two forms of HDAg have substantially distinct functions (Table 7). Extents of editing and self-cleavage have been reported to be related to the course of infection in experimental animals.

which form the outer coat of the satellite as well as that of the helper virus. This complementation is, therefore, essential for maturation, cell-to-cell spread, and transmissibility of progeny HDV virions. HDAg is the sole HDV-encoded protein consistently found in viral particles and infected cells, and it exists in two molecular species (Fig. 7) that together represent the nucleoprotein and, in addition, exert opposing regulatory functions on HDV RNA replication (Table 7). HDV is genetically unrelated to any other animal virus, including HBV. For this reason, it is classified in a separate, free-standing genus, *Deltavirus*.

Experimental propagation of HDV is readily obtained in chimpanzees infected with HBV (where HDV appears to induce more severe hepatitis than do other human hepatitis viruses) and in eastern woodchucks infected by the woodchuck hepatitis virus, which provides HDV with the required outer coat as effectively as HBV. These animal models mimic HDV infections in humans and exhibit similar liver lesions. In mice, HDV undergoes an incomplete replication due to the host's inability to support HBV growth, but this limitation has been recently overcome by the development of mouse models transplanted with human primary hepatocytes or transgenic for HBV sequences; in these, the HDV genome replicates and assembles into complete virions, although they have no tendency to spread (Dandri et al., 2006). Unlike HBV, extrahepatic replication of HDV has never been detected in infected hosts. There are no simple in vitro culture systems of diagnostic utility for HDV, although limited growth has been reported in primary hepatocytes coinfected with HBV. Also, cells can be transfected with cloned DNA containing portions of the HDV and HBV genomes to obtain clues to specific aspects of HDV genome replication. More extensive coverage of HDV structure and replication can be found in recent reviews (Taylor, 2006a, 2006b).

TABLE 7 Structure and replication of HDV: salient features

Structure
Roughly spherical, slightly pleomorphic 36- to 43-nm virions
Spherical 19-nm nucleocapsid comprising the RNA genome and ~70 copies of the only HDV-encoded protein, known as HDAg
HDAg exists in two versions with the same sequence except that the larger one (L-HDAg) has an additional 19 amino acids at the carboxy terminus compared to the short (195-amino-acid) form (S-HDAg) and undergoes posttranslational modification important for virion packaging
Outer envelope composed of cell-derived lipids and the same three glycoproteins forming the surface antigen of the helper virus (HBV)
Circular, negative-sense 1.7-kb single-stranded RNA, which folds into an unbranched rod-shaped structure due to extensive (~70%) intramolecular complementarity and base pairing (Fig. 7)
The viral RNA possesses ribozyme activity, which autocatalytically cuts itself at a specific cleavage site, and possibly self-ligating activity
Several ORFs are present in both the genomic and antigenomic strands, but only one in the latter strand is consistently expressed and codes for the HDAg
Replication
Poorly understood
Entry into cells probably mediated by the HBsAg envelope and unknown receptors (the same as for HBV?)
RNA replication takes place in the cell nucleus, presumably by a double rolling-circle mechanism involving cellular RNA polymerase II followed by site-specific autocatalytic cleavage and ligation, to generate circular genomic and antigenomic RNAs
Synthesis of S-HDAg and L-HDAg from the same ORF as a result of RNA editing (Fig. 7)
Synthesis of progeny RNA genomes regulated by the two forms of HDAg: S-HDAg transactivates, while L-HDAg down-regulates RNA replication
With the assistance of HBsAg[a], progeny genomes and HDAg assemble in the nucleus to form the virions
Release probably occurs through modalities similar to those used by HBV

[a]Whereas HDAg is mainly a nuclear protein, HBsAg is essentially cytoplasmic. They most likely interact in the endoplasmic reticulum.

The genetic variation observed in HDV is considerable, since the nucleotide sequences of independent isolates may exhibit divergences of up to nearly 40%. Based upon genetic similarity and phylogenetic analysis of a semiconserved segment of the HDAg-encoding open reading frame (ORF), three major genotypes (designated HDV-1, -2, and -3) and several subtypes (designated with letters) were initially distinguished. More recently, analysis of a large number of isolates from different geographical areas has shown that what was previously considered a subtype is in fact a distinct genotype (HDV-4) and that most African HDV isolates cluster into four additional genotypes, designated HDV-5-8 (Radjef et al., 2004). Thus, the genetic variability of HDV is much more complex than previously thought and will most likely require further reclassifications (Deny, 2006). While distinct HDV genotypes have different geographical distributions (see below), it is uncertain whether they also have a different impact on efficiency of replication and disease severity. That this may be the case is suggested by observations that genotype 2 is associated with fulminant hepatitis and adverse outcomes (cirrhosis and HCC) less frequently than genotype 1 (Wu, 2006).

The mechanism(s) whereby HDV damages hepatocytes is controversial. Interestingly, HDAg is toxic to cells when expressed in large quantities (Cole et al., 1991) but essentially apathogenic in transgenic mice expressing HDAg in the liver (Guilhot et al., 1994). In acute-phase infection, HDAg (in particular, S-HDAg) may be directly cytotoxic to hepatocytes. Later in infection, when viral replication subsides, liver damage may be essentially immunopathogenic in nature, similar to those caused by HBV and HCV (Farci, 2003).

Dynamics of HDV Replication in Infected Patients and Antiviral Immune Responses

As defined in humans and experimentally infected chimpanzees, the course of virological, immunological and clinical events accompanying HDV infection differs substantially depending on the HBV status of the host.

Coinfections

The coinfection pattern (Fig. 8, left panels) occurs when an HBV-naive individual becomes simultaneously infected with HDV and HBV as a result of exposure to a mixed inoculum. Coinfection has generally been studied in adults (typically, IDUs and other subjects exposed percutaneously to infected blood), an age group in which the helper HBV infection usually resolves in a few months, and as a result, HDV infection is also equally cleared.

In the early phases postcoinfection, the two viruses replicate acutely, but since the release of progeny HDV virions is limited by the availability of HBsAg, HDV generally becomes detectable in the circulation later than HBV. Thus, initially, the profile of infection markers is that typical of an acute HBV infection, but soon thereafter, generally shortly before the appearance of clinical symptoms and the rise of anti-HBc IgM, HDV starts to circulate in the plasma and then progressively peaks, with a delay of approximately 1 week relative to the HBV peak. Concomitant with this HDV viremia peak, a marked reduction in the titers of circulating HBV occurs that is believed to stem from inhibition of HBV expression possibly mediated by the HDAg protein (Sureau, 2006). In coinfected patients, the antibody responses to HBV are similar in dynamics and strength to those seen in patients singly infected with this virus. On the other hand, those to HDV are usually weak, often restricted to IgM, and may also be rapidly lost, so they can escape detection unless tested for repeatedly. Both viruses are generally cleared in a few months. In fact, although HDV-HBV coinfections tend to have a more severe course than infection by HBV alone, progression to chronicity remains an infrequent outcome, observed in 1 to 3% of cases. After recovery, anti-HD antibodies generally disappear within months or a few years.

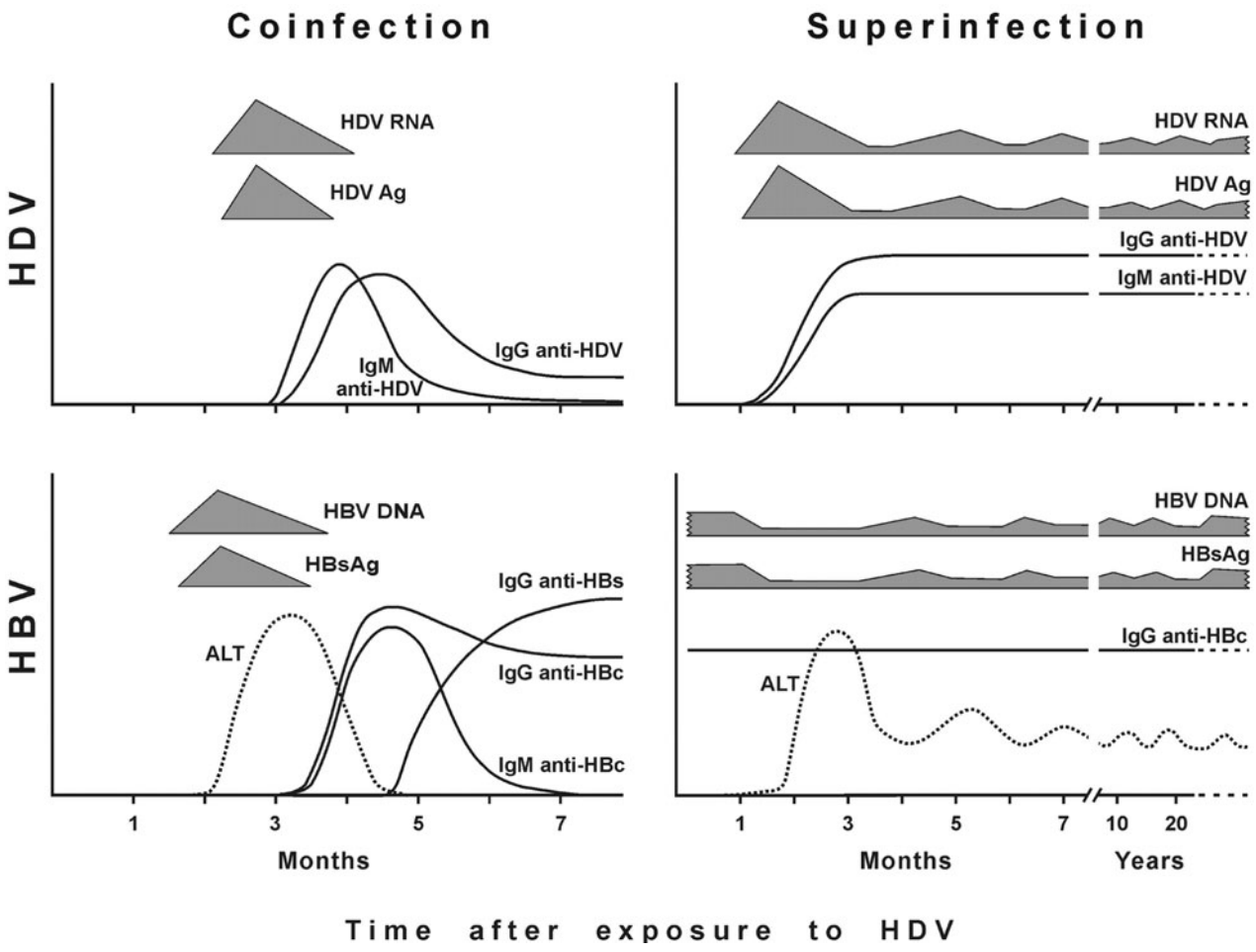

FIGURE 8 Typical courses of HDV infection. (A) Typical coinfection. (B) Typical superinfection. The major markers of the helper HBV infection also are shown. The shaded areas represent the time periods the markers indicated are demonstrable in the circulation. HDAg detectability refers to EIA methods.

The clinical outcome of HDV-HBV coinfections may range from mild to severe or even fulminant hepatitis, but complete clinical recovery is generally the rule. When present, symptoms of hepatitis develop after an incubation period of 5 to 10 weeks and, probably depending on the relative titers of the two viruses in the infecting inoculum, present a monophasic or biphasic course. In the latter case, the first bout of manifestation is generally attributable to HBV and the second to HDV.

Superinfections

The superinfection pattern (Fig. 8, right panels) is observed when HDV affects persons who are already chronically infected with HBV, and it results in persistent HDV infection in 70 to 90% of cases. Since the HBsAg necessary for productive HDV replication is already available in the hepatocytes, the course of infection is more rapid and florid than in coinfections. HDV starts to circulate in the plasma within a couple of weeks and reaches titers of up to 10^{11} infectious doses/ml. Concomitantly, a suppression of HBV viremia occurs that may be so pronounced transiently as to cause the HBsAg to be temporarily undetectable.

As judged from IgM and IgG antibody development, during superinfections, the immune responses to HDV mount more rapidly than in coinfections; however, their contribution to the natural history of HDV infection is poorly understood. As expected from the internal location of the antigen recognized, anti-HD antibodies appear unable to neutralize the virus and most probably play no role in protection, whereas anti-HBs antibodies are believed to be protective against HDV as well as against HBV. With regard to HDAg-specific cell-mediated immune responses, the limited evidence available suggests that sensitized lymphocytes may contribute to both amplification of liver injury and control of HDV replication. Indeed, the few patients who harbor HDV in the liver but have no detectable HDV RNA in blood have shown a range of anti-HDAg cell-mediated immune responses that were not found in patients with persistent viremia. The overall protective role of anti-HDV immune response is, however, still uncertain. In the woodchuck model, HDV-specific B- and T-cell responses induced by HDAg vaccination have failed to protect from HDV challenge. HDV persistence is characterized by the continuous presence of virus in the plasma and liver, although at lower levels than in the acute phase, in spite of persisting high titers of anti-HD IgG and IgM. In fact, the anti-HD IgM disappears only in rare patients who eventually also succeed in resolving the infection (Casey and Gerin, 2006; Fiedler and Roggendorf, 2006).

The changes in the preexisting clinical status brought about by HDV superinfections depend on the patient's clinical conditions. In asymptomatic HBV carriers, acute HDV replication may cause changes typical of a generally severe acute hepatitis that usually develop after a shorter incubation period (3 to 5 weeks) than in coinfections. On the other hand, in patients with symptomatic chronic hepatitis B, HDV superinfection generally produces an exacerbation of the underlying disease. Rapidly progressing subacute forms of hepatitis also have been described, and the reported overall rates of mortality have varied between 2 and 20%. Once HDV infection has become chronic, the clinical evolution is frequently toward an active chronic hepatitis of considerable gravity and an accelerated or enhanced tendency to cirrhosis and HCC (Fattovich et al., 2000; Chen et al., 2002; Sheng et al., 2007a).

Epidemiology

Similar to HBV, the only known natural host of HDV is humans. Seroprevalence surveys have shown that HDV is present worldwide and that approximately 5% of the global HBV-infected population, corresponding to 15 to 20 million people, is HDV coinfected (Farci, 2003; Radjef et al., 2004). The rates of infection vary markedly in different geographic regions. In general, HDV infects higher proportions of HBV carriers in those areas where HBV is most prevalent, but this correlation is a loose one. For example, HDV endemicity is low in many parts of Asia where HBV is widespread. The rates for HDV-seropositive HBV carriers are 20 to 60% in South America, and Central and East Asia, 5 to 20% in the Mediterranean basin, Eastern Europe, and West Africa, and 0.5 to 2% in North America and Northern Europe. In low-prevalence countries, HDV is virtually restricted to illicit IDUs, among whom it has been seen to spread rapidly, while penetrance among other categories of HBV carriers is moderate. In view of the general decline of HDV infection incidence observed in recent years (see below), IDUs have actually been identified as an important reservoir for the virus (Huo et al., 2004; Mele et al., 2007).

Except for parenteral exposure to HDV-containing blood, routes and modes of transmission are poorly understood. In particular, the transmission routes responsible for the high endemicity of certain areas are unknown, although it is widely assumed that they are the same as for HBV and are favored by poor socioeconomic conditions and poor hygiene. Sexual transmission is inefficient but has been documented in spouses of infected persons as well as among homosexual men and prostitutes, who also have been found to harbor multiple HDV genotypes as a likely result of repeated exposures. Perinatal mother-to-child transmission also has been observed. However, the cases in which no obvious risk factor can be recognized are numerous.

Most HDV isolates throughout the world belong to genotype 1. The other genotypes appear to be geographically restricted. Genotype 2 has been detected in Japan and Taiwan, and genotype 3 has been detected in South America, where the induced disease is particularly severe (Deny, 2006; Wu, 2006).

Prevention and Treatment

Potentially, all HBV carriers are at risk for contracting HDV infection. These individuals should be counseled about the additional health risk represented by HDV superinfection and on how to prevent it. Unfortunately, the populations at higher risk are also the most difficult to reach with educational campaigns. In HBV-free individuals, all of the practices that have proven useful in preventing HBV infection, primarily accurate control of blood supply and anti-HBV vaccination, have proved valuable for control of HDV spread as well. Recently, a decrease in HDV endemicity has been noted in several countries and has been attributed to the interruption of HBV transmission resulting from universal HBV vaccination, general improvement of socioeconomic conditions, and active preventive measures against promiscuity and sexually transmitted diseases (Gaeta et al., 2000; Huo et al., 2004; Mele et al., 2007). HDV is rapidly inactivated by nonionic detergents and lipid solvents, but there are few reports dealing with its resistance to inactivants.

Since HDV replication does not persist after HBsAg clearance, the treatments that lead to HBV elimination also eradicate HDV infection (Niro et al., 2005). However, hepatitis D is difficult to eradicate. IFN-α and other current treatments are at best transiently beneficial only in a small fraction of patients (Tan and Lok, 2007). Although generally proportional to the dose and duration of treatment, the response to therapy varies widely individually and sometimes occurs after discontinuation of therapy (Farci, 2006; Niro et al., 2005). Most recently, the use of pegIFN-α has demonstrated superior efficacy, leading to SVR (defined as undetectable HDV RNA and normalization of ALT levels for at least 6 months after the end of treatment) in at least 20% of patients with reduced drug dosage and side effects relative to the unconjugated drug (Castelnau et al., 2006; Erhardt et al., 2006; Farci, 2006). Ribavirin and other analogs, used as monotherapy or in combination with pegIFN-α, have shown no advantages (Niro et al., 2006). Among the drugs currently under development, the prenylation inhibitors, which block a posttranslation modification of L-HDAg essential for viral assembly, appear to be promising, since they have been highly effective at clearing HDV in animal models (Bordier et al., 2003).

Laboratory Diagnosis and Follow-Up

Performing HDV diagnostic tests in HBV-uninfected individuals is of no value. By contrast, the possible coexistence of an HDV infection is an important consideration in the management of all HBV-infected subjects, primarily if they belong to risk groups or live in countries where HDV endemicity is high. Suspicion should especially arise when the liver disease worsens or persists in spite of a negative test for HBeAg.

Laboratory diagnosis depends on the use of highly sensitive molecular techniques to detect and quantitate HDV RNA and of immunoassays that demonstrate the presence of HDAg and anti-HDV antibodies in serum. However, where HDV endemicity is low, the entire set of diagnostic tests for this virus is usually available only in a few specialized laboratories. A key to the interpretation of diagnostic tests for HDV is provided in Table 8.

Tests for HDV Antibodies

The tests for total anti-HD antibodies essentially measure those of the IgG class. In coinfections, these antibodies become detectable 3 to 8 weeks after clinical onset and can be missed due to their brief persistence. Anti-HD IgG is found both in chronic active infections and in subjects who have cleared the virus, two situations that can be distinguished by testing for HDV RNA. In chronic infections, high titers of these antibodies are considered an indicator of poor prognosis. Anti-HD IgM assays have an important role in the diagnosis of both acute and chronic infections; however, when an acute coinfection is suspected, they should be

TABLE 8 Interpretation of test results for diagnosis of HDV infection

Results[a]							Interpretation
HDV				HBV			
RNA	HDAg	Anti-HD IgM	Total anti-HD	HBsAg	Anti-HBs	Anti-HBc IgM	
+	+	–	–	+	–	+	Acute coinfection; early phase
+	–	+	+	+	–	+	Acute coinfection; late phase
+	±	±	±	+	–	±[b]	Acute superinfection
+	–	+	+	+	–	±[b]	Chronic infection
–	–	–	+	–	+	–	Past infection
–	–	–	–	–	–	–	No current infection

[a]+, positive; –, negative; ±, may be either positive or negative.
[b]Sensitive tests can be positive but at low titer.

repeated several times before an HDV infection is excluded because antibody responses to HDV are generally feeble (see above). Observations suggesting that the levels of IgM could help in identifying chronically infected patients with highly active virus replication and liver disease have not been confirmed.

Establishing whether an HDV infection results from coinfection or superinfection also has importance for the patient's management and prognosis. A useful parameter is the presence of high titers of anti-HBc IgM, which signals a recent HBV infection and is therefore strongly indicative for an ongoing coinfection (Table 8).

Tests for Detection and Measurement of HDV RNA in Plasma

HDV RNA is present in the serum of nearly all patients that are HDV positive in the liver. The presence of this marker is indicative of ongoing active HDV replication and has been found to correlate with significant liver inflammation and necrosis. The first methods for HDV RNA detection included dot and slot hybridization assays with labeled probes and had a sensitivity of approximately 10^5 to 10^6 genomes/ml plasma. These methods have been replaced by much more sensitive gene amplification methods, such as RT-PCR, which can detect as few as 100 genomes/ml. Due to their higher sensitivity and lack of interference by the patient's anti-HD response, these assays have significantly extended the duration of HDV viremia detectability in coinfections (Fig. 8) and are positive for the great majority of chronically infected patients (Farci, 2003). Furthermore, they permit the diagnosis of HDV infection in immunosuppressed patients who may fail to develop detectable anti-HD antibody. In addition, the quantitative formats of these assays provide an invaluable tool for the patient's follow-up in the course of therapy (Castelnau et al., 2006; Le Gal et al., 2005). Recently, microarrays have been developed that simultaneously detect HBV and HDV genomes and that may prove useful for blood screening (Zhaohui et al., 2004).

Tests for the Detection of HDAg in Blood

The EIA currently used for HDAg detection are positive during the preserological stage of acute infection but then become negative because the patient's anti-HD antibodies compete with those used to capture the antigen in these assays. Western blot methods for HDAg detection suffer less of this limitation and have the added advantage of separately visualizing the S-HDAg and the L-HDAg, but they are not commercially available and are labor-intensive. In coinfections with moderate or no clinical involvement, the HDAg often remains undetectable because of limited virus replication, and the infection may be signaled only by the appearance of anti-HD. The HDAg also is almost always undetectable by EIA in chronic infections.

Assays for Determining HDV Genotype

Characterizing the genotype of HDV is of limited utility in clinical practice. It is generally performed by sequencing or, more simply, by RFLP analysis of amplicons derived from the ORF that encodes the HDAg (Wu, 2006).

ADDITIONAL VIRUSES UNDER CONSIDERATION FOR POSSIBLE HEPATOPATHOGENESIS

As mentioned in an earlier section, several viruses have been and still are under scrutiny for a possible role in those forms of acute and chronic hepatitis that have no evidence of infection by known hepatitis-inducing viruses. These include GBV-C virus, TTV and related anelloviruses, and the most recently discovered NV-F virus.

GBV-C or HGV

Several years after its definitive identification, GBV-C, also known as HGV, is still a disease orphan virus. In spite of early enthusiasm that led to one of its names, its role as a possible agent of hepatitis is uncertain (for this reason, taxonomically the name GBV-C is now preferred), and there are no other clinical manifestations clearly linked to it. It is, however, well established that GBV-C is widespread in humans and can persist and circulate in the blood of infected individuals for long periods of time in the absence of detectable signs or symptoms of disease.

The Virus

In the 1960s, nonhuman primates inoculated with serum from an American physician were found to harbor a filterable agent that produced liver damage in these hosts. Samples from these animals were reexamined in 1995, using molecular approaches similar to those that had permitted HCV identification, and this resulted in the identification of three distinct but related viral genomes, one of which (GBV-C) was shown to represent a human virus. One year later, an independent group retrieved a virus from monkeys inoculated with serum from another individual with acute hepatitis and called it HGV. By comparing the genomes, GBV-C and HGV were soon recognized as different isolates of the same virus.

GBV-C is a positive-sense ssRNA virus that is presently classified within the family *Flaviviridae*. Structure and replication characteristics are poorly understood and mostly inferred from those of related viruses, especially HCV (Fig. 9 and Table 9). It should be noted, however, that the amino acid sequence homology with HCV is less than 30%. A distinguishing feature of the GBV-C genome (coding capacity for a polyprotein of 3,000 amino acids) is that the sequence encoding the capsid protein remains undefined, despite characterization of the viral particles that has clearly demonstrated a nucleocapsid indicative of the existence of such a protein. There are few reported attempts to grow the virus in vitro, and these have been essentially unsuccessful, even though limited replication has been achieved in stimulated PBMC and selected cell lines of hepatic and lymphoid origin (Polgreen et al., 2003; Stapleton et al., 2004b).

The overall genetic diversity among GBV-C isolates is limited, less than 12%. Nevertheless, it has been possible to distinguish six major genotypes, some of which have been further divided into subtypes, which have different geographic distributions. More detailed descriptions of GBV-C can be found in specialized review articles (Kiyosawa and Tanaka, 1999; Polgreen et al., 2003; Stapleton, 2003).

Dynamics of GBV-C Replication in Infected Individuals and Antiviral Immune Responses

The natural history of GBV-C in infected subjects is still poorly understood. In patients transfused with contaminated blood, the virus is found in serum after approximately 2 weeks and remains detectable throughout the duration of acute infection. Fifty to 80% of those who become infected resolve the infection within weeks, and this is associated with the development of high titers of antibodies to the envelope protein E2 (Polgreen et al., 2003; Williams et al., 2004). The remaining subjects (especially represented among the immunocompromised) become chronic virus carriers. In the latter, no E2 antibodies are detectable, and the virus circulates in blood for months or years apparently bound to low-density lipoproteins but not to antibody (Tucker and Smuts, 2001).

The sites of GBV-C replication and persistence are still poorly characterized. Even the marked hepatotropism, which was initially considered an important feature of the virus, has been strongly questioned based on low virus content and undetectability of antigenomic GBV-C RNA in the liver. In fact, GBV-C is presently believed to be primarily a panlymphotropic virus, since it replicates in the spleen and bone marrow as well as in circulating CD4 T and B lymphocytes (Tucker et al., 2000; George et al., 2006). The constant association between anti-E2 seroconversion and cessation of infection indicates that E2 antibodies are of utmost importance for virus clearance. The existence of anti-E2-positive individuals who appear to reactivate a previous infection or become superinfected by a different strain of the virus has led to the suggestion that these antibodies do not possess direct virus-neutralizing activity. This inference is, however, contradicted by the recent development of monoclonal antibodies to E2 that inhibit virus binding to cells (McLinden et al., 2006). In any case, it is likely that anti-E2 acts in concert with other hitherto undefined antiviral effectors. A role for cell-mediated immunity in infection control has been hypothesized but remains to be investigated.

Although GBV-C was initially associated with acute and chronic liver pathologies, including biliary duct inflammation and fulminant hepatitis occurring in patients with no other known etiology (Halasz et al., 2001), an unequivocal demonstration that it is indeed implicated in at least a subset of these pathologies is lacking. Conversely, there are numerous reports of acute and chronic GBV-C infections occurring in individuals with no biochemical evidence of liver damage. Moreover, in the few patients infected with GBV-C alone who present liver enzyme elevations, these are usually modest and temporally unrelated to GBV-C viremia fluctuations. Even in patients infected with HBV or HCV, a concomitant infection with GBV-C does not seem to increase liver damage or affect the outcome of therapies (Schwarze-Zender et al., 2006). However, also in view of the fact that the virus causes mild hepatitis in inoculated macaques (Ren et al., 2005), it cannot be excluded that GBV-C may occasionally produce moderate liver disease (Couzi et al., 2004).

Recently, interest in GBV-C has been invigorated by reports that HIV-infected patients who also are chronically coinfected with GBV-C have reduced HIV levels, higher CD4 T-cell counts, slower disease progressions, and improved

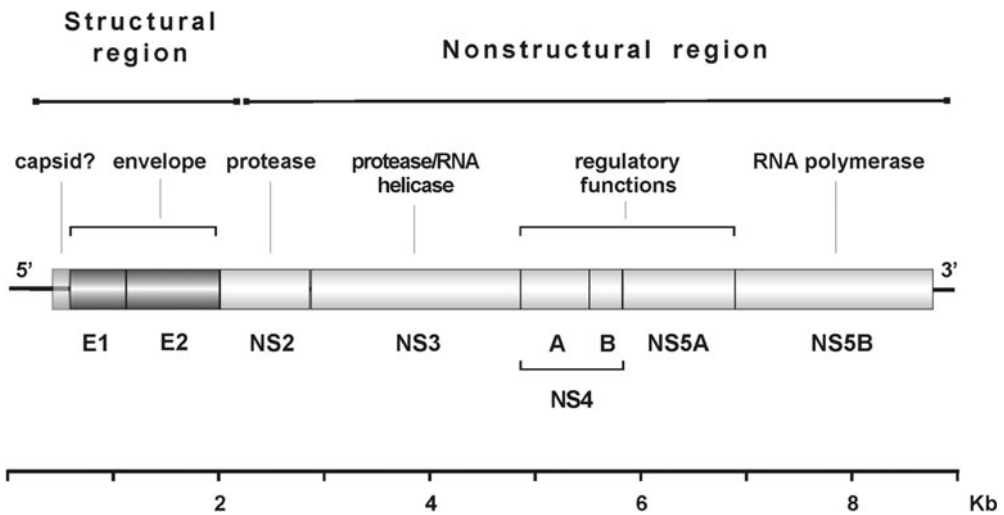

FIGURE 9 Organization of the GBV-C genome and encoded proteins (Table 9). Capsid?, the sequence encoding the capsid protein is still undefined; E, envelope; NS, nonstructural.

TABLE 9 Structure and replication of GBV-C; known and inferred properties

Structure
Spherical, 40- to 60-nm-diameter virions
Small or absent capsid protein
Pericapsid or envelope composed of two or three virus-coded proteins
Linear, positive-sense, 9.4-kb ssRNA
Genome organized in structural (E1, E2, possibly core, and p7) and nonstructural (NS2, NS3, NS4, and NS5) coding regions and two highly conserved noncoding regions (UTRs) located at each terminus; an expressed ORF is also present in the antigenome (Fig. 9)
No hypervariable domains detected in the genome
Replication
Enzymatic activities apparently coded: two proteases, helicase, RNA polymerase
A minus RNA strand complementary to the viral genome can be detected in infected cells
Cap-independent synthesis of a polyprotein
Polyprotein cleaved by viral and possibly cellular proteases

responses to antiretroviral therapies relative to singly HIV-infected patients (Stapleton et al., 2004b; Kaiser and Tillmann, 2005; Xiang et al., 2005). Although there are reports that contradict these intriguing observations (Sheng et al., 2007b), an inhibitory effect of the E2 protein of GBV-C on HIV-1 entry into cells has recently been described (Jung et al., 2007).

Epidemiology

Active GBV-C infection, defined by the presence of variable viral burdens in plasma, is present in the general population worldwide at rates varying between <1% and 4% in different countries. By contrast, surveys have found E2 antibodies in 2 to 20% of healthy adults, showing that many individuals experience an inapparent self-limited infection at some time during their life (Wiwanitkit, 2005).

That high GBV-C prevalence rates have been detected in polytransfused patients, IDUs, and individuals at risk for parenteral infection in general is clear evidence that the infection is often blood borne. However, the presence of the virus in individuals not at risk for such transmission and the fact that the virus is shed in saliva and other biological fluids suggest that additional routes of transmission are possible. Indeed, the high frequency of GBV-C infection in HIV-, HBV-, and HCV-infected patients supports the idea that these infections disseminate in similar manners. Sexual transmission is considered common, and male-to-male sex has actually been proposed as the most effective means of GBV-C spread (Berzsenyi et al., 2005). Vertical transmission is rare (Li et al., 2006).

Prevention and Treatment

In theory, the strategies for prevention of GBV-C infection should proceed along lines similar to those used in prevention of HIV-1, HBV, and HCV infection. Whether blood donations should be screened for GBV-C genomes and/or antibody is, however, a matter of debate. At present, the cons are judged to outweigh the pros, but with the development of more standardized, reliable, and inexpensive GBV-C diagnostic methods and increasing concern for blood supply safety, the matter should be reconsidered. There are no antivirals of proven efficacy, and what we know about GBV-C pathogenicity would not justify specific clinical trials. In patients given IFN-α alone or in association with ribavirin for HBV or HCV, these treatments have abated concomitant GBV-C viremia, but the effect was generally transient (Schwarze-Zender et al., 2006).

Laboratory Diagnosis of GBV-C Infection

Diagnosis of GBV-C infection is usually carried out by testing plasma or serum for the viral genome using molecular methods and/or for antiviral antibodies. It should be noted, however, that the conditions for which performing such tests is deemed clinically useful are limited. In fact, at present, the only patients considered worth testing for GBV-C are those with presumed viral, acute or chronic liver pathologies that are now designated as non-A to -E. As a consequence, the assays are not offered by all laboratories.

Tests for the Detection and Measurement of GBV-C RNA in Plasma

Various molecular methods have been proposed for detecting GBV-C RNA in plasma and other biological specimens, and some are commercially available. Early RT-PCR protocols utilized primers designed on coding regions of the viral genome, but current methods target the more conserved 5′ UTR (Souza et al., 2006). Standardization is still inadequate, however, and amplification using different primer sets should be systematically performed to confirm the validity of the results. In recent studies, differing rates of GBV-C RNA positivity were found when the same sera were tested with different sets of primers, and reproducibility among laboratories was found to be surprisingly low, even when the tests were carried out with the same commercial assay (Bortolin et al., 2004; Souza et al., 2006). Methods for quantifying GBV-C using real-time technologies also have been developed, but they appear to be of even less clinical utility than the qualitative assays, since there is no evidence that high viral loads impact the host more than low viral loads. At present, GBV-C genotyping is performed for epidemiological purposes only. It is generally carried out by characterizing amplicons of the 5′ UTR by direct sequencing or RFLP analysis.

Testing for GBV-C Antibodies

Antibodies to GBV-C can be determined with the use of several structural and nonstructural proteins of the virus. However, the only commercial assay currently available is an enzyme-linked immunosorbent assay (ELISA) based on recombinant E2 protein. Other tests, including assays using chimeric and cyclic synthetic oligopeptides reproducing stretches of different nonstructural viral proteins, have been developed but have yet to be validated. Recently proposed protein microarrays for the contemporaneous detection of multiple antigens and antibodies of hepatitis viruses, including IgG against GBV-C (Xu et al., 2007), seem an interesting

TABLE 10 Interpretation of diagnostic test results for GBV-C infection

Results[a]		Interpretation
Anti-E2 antibody	GBV-C RNA	
–	+	Acute or chronic infection
+	+	Resolving infection, reactivation, or chronic infection
+	–	Past infection
–	–	No infection

[a]+, positive; –, negative.

approach worthy of further investigation. Confirmatory tests suggested for verifying a positive ELISA result include a line probe assay and a sandwich ELISA but are impractical for routine use. As summarized in Table 10, the uncertainties existing in the interpretation of currently available GBV-C diagnostic test results are still considerable. In part, this is also due to the fact that attempts to develop sensitive and specific assays for anti-GBV-C IgM have so far been frustrating.

TTV and Related Anelloviruses

TTV was discovered in the serum of a patient with post-transfusion non-A to -G hepatitis by Japanese workers in 1997. Since then, not only has TTV been shown to be extremely common worldwide and to display a remarkable genetic variability—the virus has been subdivided into 5 widely diverse genogroups and over 40 genotypes—but a number of related yet clearly distinct viruses have also been identified. The latter include torquetenominivirus (TTMV) and torquetenomidivirus (TTMDV), both of which have smaller genome sizes relative to TTV (Takahashi et al., 2000; Ninomiya et al., 2007). All of these agents are currently classified in a free-standing genus, *Anellovirus*, which is expected to be soon upgraded to the rank of family. Similar to GBV-C, they are known to be transmitted though blood or blood products, but none has yet been linked with certainty to any human disease, including hepatitis. The discussion below is almost exclusively limited to TTV, since this is the only anellovirus which has been investigated to a reasonable extent.

The Virus

Although knowledge of the genomic features of anelloviruses has been growing rapidly, lack of important tools, such as sufficiently sensitive in vitro culture systems and suitable methods for analyzing the viral antigens, has hitherto prevented a satisfactory understanding of their morphology and physicochemical properties. Based on genome properties analogous to other single-stranded DNA viruses and limited direct evidence, TTV particles are currently believed to have the features listed in Table 11. In particular, the genome (Fig. 10) is a circular ssDNA molecule of ~3.8 kb in TTV versus ~3.2 kb in TTMDV and ~2.8 kb in TTMV.

Limited replication of TTV has been reported in cultures of polyclonally activated human T lymphocytes. The attempts to infect experimental animals have been few and are hampered by the demonstrated presence of autochthonous anelloviruses in many animal species (Okamoto et al., 2002). The antigens of TTV are also essentially unexplored, as yet. However, given the great diversity in the coding regions of different TTVs, a great antigenic diversity is most likely.

Dynamics of TTV Replication in Infected Individuals and Antiviral Immune Responses

The natural history of TTV infection is poorly understood. However, it is well established that the virus generally persists in infected patients for protracted periods of time or indefinitely. Chronically infected persons have been found to carry TTV for up to 22 years (Matsumoto et al., 1999) and to produce and clear 10^{10} to 10^{11} virions per day (Maggi et al., 2001). They have also been reported to harbor high levels of TTV DNA in many body sites, thus implying that TTV is polytropic in nature; however, by analogy with other ssDNA viruses, it is likely that only cycling cells support significant virus replication (Bendinelli and Maggi, 2005). Cases of self-limited infection also have been described (Tsuda et al., 1999; Bendinelli et al., 2001), but it is uncertain whether they reflect true virus eradication or rather declines of the virus content in blood under the threshold of the virus detection methods used.

TABLE 11 Structure and replication of TTV, the prototype of anelloviruses: known and inferred properties

Structure
- Roughly spherical, about 30-nm-diameter virions with ~1.32 g/cm^3 buoyant density in CsCl
- No pericapsid or external lipid envelope
- Circular, negative-sense, single-stranded DNA ranging in size from 3.5 to 3.8 kb (Fig. 10)
- Genome consisting of a large coding region (~2.6 kb divided into two major ORFs and a few smaller partially or totally overlapping ORFs) and a relatively short UTR
- The UTR encompasses an 80- to 160-nt sequence with a high guanine and cytosine content, several regulatory elements believed to be important in replication, and a short domain highly conserved among all anelloviruses
- The ORF-1 of some isolates contains a central hypervariable domain

Replication
- Poorly understood
- DNA replication is believed to take place in the nucleus, probably by a rolling cycle mechanism involving virus-coded proteins
- Three to four species of viral mRNA, transcribed from the negative strand and having different lengths but common 5′ and 3′ termini
- Modes of virion assembly and release from producer cells unknown

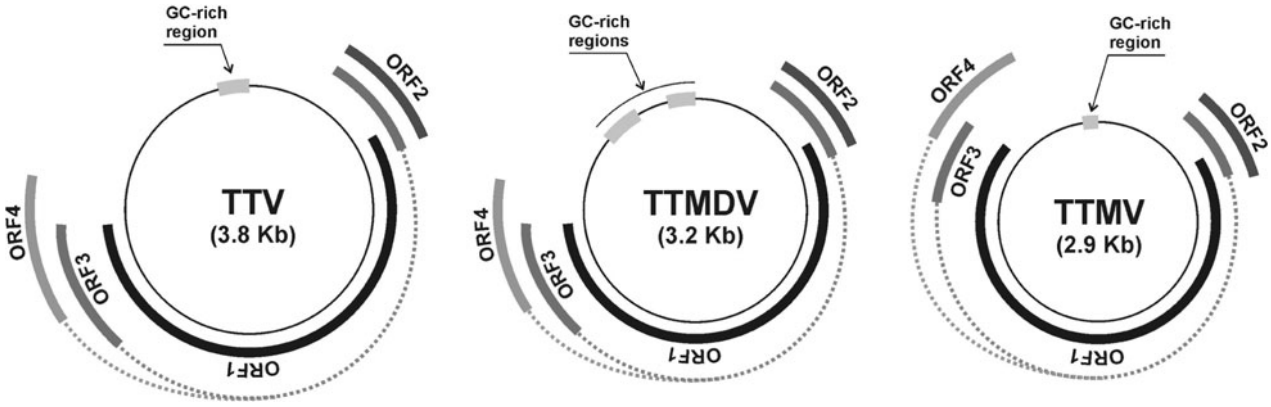

FIGURE 10 Genome organization of TTV and the related anelloviruses TTMV and TTMDV. The arcs represent the major ORFs identified. Several smaller ORFs may also exist.

Although it is clear that infection evokes a specific antibody response, the immune responses generated by TTV have been investigated very little. Most virions circulating in the blood of chronically infected subjects have been seen to be bound to IgG, and TTV-specific IgM and IgG were detected in patients infected through a virus-contaminated transfusion (Ott et al., 2000; Tsuda et al., 2001). Clearly, however, these responses are unsuccessful at overcoming the infecting virus. The demonstration of superinfections in subjects sequentially exposed to different strains of TTV also suggests that protective immunity is either nonexistent or has a limited breadth.

As mentioned, the medical significance of the anelloviruses is still controversial. Although it cannot be excluded that certain TTV isolates may be associated with transient and mild increases of liver enzymes (Nobili et al., 2005), the early suggestion that TTV might be an important cause of cryptogenetic hepatitis has not been corroborated. A possible role in the genesis of other diseases of unknown origin has, therefore, been sought. Given the chronic nature of most if not all TTV infections, attention has been especially focused on chronic diseases such as rheumatoid arthritis, systemic lupus erythematosus, and certain neurological diseases for which a viral etiology has long been suspected; the findings so far have, however, been inconclusive (Hino and Miyata, 2007). Since TTV infection is acquired early in life (see below), attempts are also underway to correlate it with acute pathologies of presumable viral origin occurring in infants. These studies have shown that TTV replicates in the respiratory tract, where it might be the cause of at least some acute respiratory diseases (Biagini et al., 2003) and also act as a copathogen in the genesis of asthma and bronchiectasia (Maggi et al., 2003a; Maggi et al., 2003b; Pifferi et al., 2005; Pifferi et al., 2006). However, until these and other findings have been independently confirmed, anelloviruses should essentially be considered "viruses waiting for a disease."

Epidemiology

TTV and other anelloviruses are extremely ubiquitous worldwide. Indeed, in the recent literature, prevalence rates of TTV viremia of 80% or more in the general population are quite common, regardless of age, gender, socioeconomic conditions, and geographic location. Moreover, genogroup 1 and 3 TTV are more prevalent than other genogroups, and geographical differences probably exist in their distributions (Maggi et al., 2005; Devalle and Niel, 2004). Also, the majority of individuals harbor multiple genetic forms of TTV in the bloodstream (Maggi et al., 2005), indicative of high contagiousness. Indeed, the fact that TTV is shed in great abundance with many bodily materials, including saliva, nasal fluids, feces, genital secretions, and breast milk suggests that blood-borne transmission is only one of many ways TTV can spread interpersonally and that there may be quite a few ways TTV can circulate in the population. Evidence also has been reported that TTV can be transmitted transplacentally. There is no evidence that the TTV-like viruses detected in animals are contagious for humans, suggesting that they might be species specific. Finally, it should be noted that chronic TTMV is possibly more widespread than TTV (Biagini, 2004; Biagini et al., 2006; Thom and Petrik, 2007) and that the same appears to be true for TTMDV.

Prevention and Treatment

A recent report has shown that TTV can be especially hard to remove from commercial blood derivatives, where it is usually present in high titers. There are no reports directly investigating the sensitivity of TTV to antivirals. However, TTV viremia was found to remain unchanged or to decline only transiently in hepatitis C patients given standard treatments for this disease (Maggi et al., 2001).

Laboratory Diagnosis of TTV Infection

Since TTV has not been firmly associated with any clinical manifestation, the practical utility of performing a diagnosis of acute or chronic infection is questionable. At present, it seems reasonable to limit the tests to those patients with hepatitis in whom all the other possible causes of infection have been excluded.

The only diagnostic approach currently available is detection of the viral DNA in plasma or other clinical specimens. The methods described include several formats of PCR targeted to different regions of viral genome; however, the one potentially capable of amplifying all of the genetic forms of TTV hitherto recognized is based on a small, highly conserved segment of the UTR. Used in real-time format, this "universal PCR" has led to a correct appreciation of the pervasiveness of TTV and revealed that, in individual subjects, its loads may vary between 10^3 and 10^8 DNA copies/ml of plasma (Maggi et al., 2001; Maggi et al., 2005). Positive specimens may be further analyzed for the genogroup(s) of TTV present by genogroup-specific PCRs and/or sequencing (Maggi et al., 2005). Due to frequent identification of

new viral genotypes, the primers of these assays (as well as those for TTMV and TTMDV) need to be continuously updated to expand their detection breadth.

NV-F

A novel virus designated NV-F was recently identified by Taiwanese workers in an acute non-A, non-E hepatitis subsequent to colon surgery and transfusion, by subjecting the patient's serum to nonspecific DNA amplification, followed by elimination of chromosome-derived sequences (Yeh et al., 2006). Preliminary surveys using specifically designed PCRs have shown a high prevalence of NV-F DNA in patients with non-A, non-E hepatitis and in HBV- and HCV-infected people. NV-F DNA also has been detected in 12 of 78 HCC tissue specimens. Further, an ELISA using a virus-derived synthetic peptide has revealed antibodies in many of those who harbored the virus, and an antiserum generated with a recombinant peptide has demonstrated the virus in the hepatocytes of infected patients (Hsu et al., 2006; Yeh et al., 2006; Yeh et al., 2007). The viral genome is ssDNA, otherwise the virus is completely uncharacterized at the time of writing. Future studies will determine if it is a significant cause of hepatitis.

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Rabies

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22

Rabies is a viral infection of the central nervous system (CNS) of mammals, caused by a neurotropic virus. The disease is maintained in host populations of canines, other terrestrial carnivores, and bats. Transmission is through infectious saliva, transferred by bites of clinically rabid animals to other susceptible hosts. The disease is characterized by a long and variable incubation period followed by acute, progressive encephalitis culminating in the death of nearly every infected animal.

Modern methods provide for the prevention of rabies infection in humans and animals by pre- or postexposure vaccination. Elimination of rabies has been achieved in canine populations over large areas by widespread dog vaccination programs combined with dog control activities. The disease has even been controlled in some wildlife populations by the effects of campaigns to vaccinate the main vector using distribution of baits containing vaccines that are effective by ingestion. However, the disease still exacts a horrible toll in terms of human and domestic animal mortality in large areas of the world, particularly where resource limitations prevent the implementation of these prophylactic and control measures. A truly widespread pathogen, rabies virus has been detected in nearly every mammalian order (Rupprecht et al., 2002). Rabies has been found on every continent except Antarctica (Warrell and Warrell, 2004). Still reportedly accounting for at least 55,000 deaths in humans annually worldwide (WHO, 2005), it also is associated with enormous opportunity costs where extraordinarily expensive programs must be initiated and maintained to protect humans and domestic animals.

Although there are numerous early historic references to rabies in wildlife throughout the world, the zoonotic potential of the disease always was and remains exemplified by its presence in domestic dogs. Where rabies has been controlled in dogs, human mortality from the disease has promptly become uncommon. However, because human populations are not natural hosts of rabies virus, the disease in humans is incidental to the reservoir of the disease in wild and domestic animals. Consequently, a more appropriate measure of the impact of rabies on public health in the areas where canine rabies has been controlled is the estimate of the prevalence of the disease in animal populations and the expense involved in preventing transmission to humans (Smith and Seidel, 1993). Expenditures for preventive rabies treatments for humans and domestic animals in the United States exceed $100 million annually (Uhaa et al., 1992). Since the last edition of this manual, there has been a single survival of a human rabies patient following a novel treatment attempt as well as two tragic multiple-transmission events as a consequence of organ transplant.

HISTORY

Several authors have produced exhaustive and fascinating summaries of the history of rabies and humans (Theodorides, 1986; Steele and Fernandez, 1991; Baer et al., 1996). Rabies has been one of the most widely and consistently feared zoonotic diseases since the earliest recorded human history. The transmission of rabies to humans by the bite of mad dogs was included in the Eschunna Code of ancient Mesopotamia in the 23rd century B.C. Greek mythology included a god whose task it was to counteract the effects of rabies. Aristotle's *Natural History of Animals*, written in the 4th century B.C., described a "madness" in dogs, and went on to say, "this causes them to become very irritable and all mammals they bite become diseased." Note that by this philosopher's time it was already observed that rabies was a disease of mammals, transmitted by the bite of the afflicted animal, resulting in behavioral changes, including aggression.

Earliest methods of treatment for the prevention of rabies included submersion of the patient in water, cautery of the wound with heat or caustics, wound treatment with the pharmacological ideas of the time (Baer et al., 1996), and systemic treatment with an astounding variety of substances of animal and herbal origin, including the "hair of the dog that bit you." Subsequent developments in the prevention and diagnosis of rabies have either led or paralleled human accomplishments in the sciences of infectious diseases. Georg Zinke in 1804 discussed the "agent of rabies" and described experiments that demonstrated the path of the virus from saliva to wound to saliva (Wilkinson, 1988). During the period 1881 to 1884, Louis Pasteur described the involvement of the CNS and salivary gland in the disease and discussed attenuation of the virus and the theoretical basis of immunizing injections. In 1885, he reported the landmark first human postexposure vaccination of Joseph Meister, and by 1896, he reported on the treatment of 350 exposed persons, of which only one developed rabies.

Successful canine vaccination campaigns became a reality with the development of modified live virus vaccines by serial chick embryo passage (Koprowski and Cox, 1948). The severe reactions that too frequently accompanied exposure to nervous tissue-derived human vaccines were avoided with the development of cell culture vaccines after fixed rabies virus was adapted to human diploid cell cultures in 1965 (Wiktor and Koprowski, 1965). Even with potent vaccines, an efficacy approaching 100% for postexposure treatment (PET) of humans was regularly achievable only when it was recognized in 1945 that immune serum given at the site of the bite was also critical to survival (Habel, 1945).

The diagnosis of rabies entered the modern era in 1903 when Negri discovered intracytoplasmic inclusions in large nerve cells of rabid animals. These inclusions, pathognomonic for rabies, would bear his name and be the basis for rabies postmortem diagnosis for more than half a century (Negri, 1903). The development of the mouse inoculation test in 1935 (Webster and Dawson, 1935) introduced a reliable diagnostic and research virus isolation tool, upgraded dramatically in speed beginning in 1978 by the application of in vitro cell culture techniques (Smith et al., 1978). The application of the direct fluorescent antibody test to rabies examination added modern sensitivity, specificity, and rapidity to rabies laboratory science (Goldwaser and Kissling, 1958). The application of monoclonal antibody methods to rabies virology (Wiktor et al., 1980) revolutionized the study of rabies and rabies-related viral antigenic relationships. Molecular methods based on PCR technology (Saiki, 1989) were applied to the detection of lyssaviruses, the genus in which rabies is classified, in the early 1990s (Sacramento et al., 1991) and have been utilized in an increasingly diverse role in rabies research and molecular epidemiology and to a lesser degree in rabies diagnosis (Trimarchi and Nadin-Davis, 2007).

BIOLOGY OF THE VIRUS

Rabies is the prototype virus of the *Lyssavirus* genus of the order *Mononegavirales*, family *Rhabdoviridae* (Wunner et al., 1995). The *Mononegavirales* are characterized by a nonsegmented, negative-stranded RNA genome, encapsulated tightly into a ribonucleocapsid structure. The *Rhabdoviridae* are classified as a group based on a similar conical or bullet-shaped appearance by electron microscopy. The host range for the family is highly diversified, including plants, arthropods, fish, and mammals. Previously known simply as the rabies and rabies-related virus group (Shope et al., 1970), the genus *Lyssavirus* is presently composed of genotype 1, classical rabies virus, and six other genotypes that are closely related antigenically and genetically and cause a clinical disease indistinguishable from rabies (Tordo et al., 2004). Genotype 1 includes the majority of field viruses of global distribution in terrestrial mammals and in insectivorous and hematophagous bats of the Western Hemisphere as well as the laboratory and vaccine strains (WHO, 1994). Distribution of the nonrabies lyssaviruses is restricted to the Old World. They include genotype 2, Lagos bat virus, isolated from African bats; genotype 3, Mokola virus, isolated from African rodents; genotype 4, Duvenhage virus, isolated from African bats; genotypes 5 and 6, European bat lyssaviruses 1 and 2, isolated from European bats (Kissi et al., 1995); and genotype 7, Australian bat lyssavirus, isolated from Australian bats (Hooper et al., 1997). There are four lyssaviruses recently identified in Eurasian bats that are candidates to become additional genotypes (Hanlon, 2005). Using an analysis based on rates of mutations and extrapolating back over centuries to assign timeframes to molecular genetic trees, Badrane and Tordo (2001) inferred that chiropteran hosts were the initial lyssavirus reservoir. Lyssaviruses are serologically distinct from other rhabdoviruses (Shope and Tesh, 1987).

The rigid structure of the rabies virion measures approximately 180 nm by 75 nm (Hummeler et al., 1967; Vernon et al., 1972). The virus is hemispherical at one end and usually planar at the other end, where it buds off last from the surface membrane of an infected cell. The particle contains a helical nucleocapsid surrounded by a lipid bilayer envelope. Spike-like surface projections protrude 10 nm from the outer surface of the lipid bilayer. Disruption of the virus discloses five proteins. The internal ribonucleoprotein complex (RNP) contains the viral RNA associated with three internal proteins: a large (190-kDa) transcriptase, or L protein; a 55-kDa nucleoprotein, N; and a 38-kDa noncatalytic polymerase-associated phosphorylated protein, P. The viral envelope is composed of a 26-kDa matrix protein, M, and an envelope sheath consisting of lipids derived from the host cell plasma membrane and the surface spikes formed by a 67-kDa glycoprotein, G (Wunner et al., 1988).

The rabies virus genome consists of a single-stranded, nonsegmented RNA molecule of negative-sense polarity. It is approximately 1,200 nucleotides in length and has a molecular mass of approximately 4.6×10^6 kDa. The viral RNA is transcribed into five polyadenylated, monocistronic mRNA species, corresponding to the five viral proteins. The negative polarity of the rabies genome prevents direct translation into viral proteins, requiring an autonomous transcription step facilitated by the RNA polymerase (Tordo, 1996), and the genome is therefore not infectious. This virion-associated RNA polymerase is "error prone," and there is an absence of RNA proofreading, repair, and post-replication error correction mechanisms in the cell. The infidelity of the RNA polymerase of negative-strand RNA viruses is the main cause of nucleotide misincorporation in genome RNA, generating RNA sequence heterogeneity (Wunner, 2007).

The development of PCR gene amplification and direct nucleotide sequence analysis (Sacramento et al., 1991) has substantially accelerated progress toward the understanding of the structure-function relationships of the various elements of the rabies virus (Tordo, 1996). The RNP functions in the transcription and replication of the virion. Accumulations of RNP constitute the intracytoplasmic inclusions in infected cells, which have diagnostic importance because they can be detected by direct observation with histologic methods and by antigen detection methods employing N protein-specific antibodies. The G protein is the viral antigen that induces the production of virus-neutralizing antibodies, conferring immunity against exposure (Wiktor et al., 1984). The induction of antibody and the conferred immunity is dependent upon the intact secondary and tertiary structure of the G protein (Koprowski, 1991). The G protein is responsible for the attachment of the virus to the cell and the properties determining its transport in a retrograde fashion into the CNS (Lafon, 2005).

Rabies virus is synthesized in the cytoplasm of infected cells and is released by budding through cell membranes (Murphy, 1986). The virus is somewhat resistant to air drying and freeze-thaw cycles and is relatively stable at pH 5 to 10. However, it is labile to pasteurization temperatures and UV light as well as lipid solvents, ethanol, iodine disinfectants, and quaternary ammonium compounds (Kaplan, 1996).

PATHOGENESIS AND PATHOLOGY

All mammals are susceptible to rabies infection. With very rare exceptions, rabies infection terminates in the death of each infected individual. Rabies virus has evolved a pathogenesis within the individual animal that facilitates the maintenance of the virus in the true host, the reservoir species population. Maintenance of the virus in the reservoir population by direct host-to-host transmission is dependent on simultaneous infection of the brain and salivary glands. It is the impact on behavior resulting from infection of the limbic system that induces biting behavior, and concomitant infection of the salivary gland tissue allows infectious doses of virus in the saliva to serve as an infectious inoculum for bite transmission. This pathogenic pattern has permitted the entrenchment of the virus in host populations and the continued risk to humans of exposure (Murphy, 1986).

Empirical and laboratory evidence accumulated over centuries related to rabies cycles vectored by domestic and wild species support the conclusion that rabies is transmitted by entrance of the virus by bites of rabid animals. While aerosol transmission is sometimes considered a possible mode of transmission of rabies to humans, very little data support such a conclusion. There has been apparent nonbite transmission to humans and animals by introduction of the virus by inhalation of infectious aerosols in a very unusual bat cave environment in the southwestern United States (Constantine, 1962). Empirical data in this report verified aerosol transmission to caged animals in this cave environment. However, the conclusion that the two associated human cases in that setting were definitely the result of exposure to airborne virus may need reconsideration in light of current observations that bat bites capable of rabies transmission may be associated with limited injury (Rupprecht, 1996; Gibbons, 2002). Rabies transmission to humans has been reported to have occurred by aerosol in two laboratory accidents (Winkler et al., 1973; CDC, 1977). In both cases, the infected individuals worked with rabies virus in a setting that could have allowed alternative means of infection. In one study using recent technologies, experimental aerosol transmission of rabies virus was documented in mice but failed to occur in two species of bats (Davis et al., 2007). Transmission by direct contamination of mucous membranes by saliva also has been reported (Afshar, 1979). Infection by ingestion of infected tissues was reported in dogs feeding on infected fox carcasses in the Arctic (Mansel, 1951) and in numerous laboratory studies (Charlton, 1988). Human-to-human transmission has been reported by bite, mucous membrane exposure (Fekadu et al., 1996), and as a result of corneal transplants from rabies victims in eight occurrences worldwide (CDC, 1999a). In 2004, fatal rabies infections occurred in Texas in four recipients of solid organs from a single donor (Srinivasan et al., 2005). The donor retrospectively was determined to have been infected with rabies of bat origin (Krebs et al., 2005) at the time of his death. In the following year, three fatal rabies cases occurred in recipients of organs from a single donor in Germany (Hellenbrand et al., 2005; Johnson et al., 2005).

When an animal becomes infected following exposure from the bite of an infected animal, the virus may invade peripheral nerves or nerve endings directly or may first be "amplified" by invasion of striated muscle cells prior to infection of the nerve endings (Charlton, 1988). It is not clear if the infection of myocytes at the site of exposure is an essential aspect of the pathogenesis of rabies or how this growth contributes to long incubation periods. The early events of viral replication and muscle and nerve cell infection at the site of exposure occur without substantial stimulation of the immune system. The binding of rabies virus to the host cell is mediated by the viral G protein. The rabies receptor is complex and may vary with cell type. The putative receptor in muscle cells is the nicotinic acetylcholine receptor (Lentz et al., 1984; Baer and Lentz, 1991; Lewis et al., 2000), but there is evidence that the neural cell adhesion molecule CD56 (Thoulouze et al., 1998) and the low-affinity neurotrophin receptor (p75NTR) (Tuffereau et al., 1998; Langevin et al., 2002) may also serve as rabies virus receptors. It is likely that the virus is not limited to a single receptor for infection of mammalian cells (Lafon, 2005; Wunner, 2007). Cell entry may be by fusion of the viral envelope with the cellular membrane (Superti et al., 1984) or through coated pits and uncoated vesicles (Tsiang et al., 1993). After entering sensory or motor nerve endings, its genome progresses centripetally transneuronally, only by retrograde axoplasmal flow, to the CNS (Kristensson and Olsson, 1973) and similarly within the CNS from first-order neurons to second-order neurons (Kelly and Strick, 1997). The nervous-tissue pathway to the CNS and lack of a viremia may limit exposure to the immune system, explaining the lack of an early antibody response (Krebs et al., 1995).

Virus replication in the CNS occurs mainly in neurons, with extensive distribution in the brain and spinal cord. Rabies virus is described as nonlytic, as it does not generally cause host cell destruction. However, an inverse relationship between the induction of apoptosis (natural cell death) with the pathogenicity of rabies virus strains suggests that apoptosis may be a protective rather than pathogenic mechanism (Jackson et al., 2006). Recognizable clinical signs of rabies generally do not appear until several replication cycles have occurred in the brain (Kaplan, 1985). Centrifugal spread occurs simultaneously via anterograde axoplasmic flow from the CNS to peripheral nerves and to some nonnervous tissues, including, most importantly, the salivary glands. This accounts for the appearance of rabies virus in some tissues and fluids up to a few days before recognized onset of rabies symptoms (Charlton, 1988). Although infectious doses of virus in the saliva of vectors at times of aggressive behavior is paramount for the maintenance of the virus in host populations, virus presence in saliva may be sporadic during and just prior to the clinical period (Constantine, 1967; Fekadu et al., 1982). Terminally, rabies antigen may be demonstrated in many tissues, including the buccal, nasal, and intestinal mucosa, urinary bladder, epidermis, cornea, lungs, kidneys, adrenal medulla, and brown fat (Debbie and Trimarchi, 1970). Virus in these nonsalivary tissues has not been shown to be responsible for host-to-host transmission of epidemiologic significance.

Studies in animal models have demonstrated that immune mechanisms are involved in the neuropathogenesis of rabies. There have been numerous observations that animals and humans that die despite some exposure to rabies vaccine prior to or immediately after exposure to rabies often succumb with a shorter incubation period than naive individuals (WHO, 1984). In an in vitro model using a mouse macrophage cell line, the presence of rabies-neutralizing antibodies in concentrations below protective levels actually enhanced the ability of rabies virus to infect these cells (King et al., 1984). Immunosuppression has been demonstrated to have a sparing effect in some situations (Smith et al., 1982). Furthermore, virus replication may suppress production of cellular neuropeptides and neurotransmitters, leading to the functional CNS failure and the fatal outcome of rabies infection (Fu, 1997).

The classical rabies pathogenesis described above results in a variable but long incubation period, typically 10 days to several months but rarely of many years' duration (Smith et al., 1991). The incubation period is followed by an acute, undelayed progression of disease following onset through the classical rabies encephalitic stages, culminating in death. Other observed pathogenic patterns exist, but they are extremely uncommon in naturally occurring disease. These rare and atypical pathologies may include shorter or longer incubation periods (less than 10 days or up to many years), prolonged clinical period, variations in excretion of the virus, recovery with or without chronic disability, and a carrier state of virus shedding without clinical manifestations. Infection with an Ethiopian strain of rabies virus was demonstrated to result in some dogs that shed virus in their saliva for long periods without clinical manifestations (Fekadu et al., 1981). A population of hyenas in the Serengeti has been shown recently to have a high rate of rabies seroprevalence (37%). Half of the antibody-positive hyenas had evidence of rabies virus RNA in their saliva, yet no infectious virus was demonstrable in saliva and rabies-like clinical manifestations were not observed (East et al., 2001). However, no convincing evidence of a carrier state exists in naturally occurring rabies in North America (Charlton, 1988).

CLINICAL RABIES

Following the variable incubation period, the disease in humans is marked by a brief prodromal period of several days' duration, consisting of complaints of nonspecific symptoms including malaise, anorexia, fatigue, headache, and fever. Characteristically, during this period there is pain and paresthesia, or "tingling" at the site of exposure, which are usually the first rabies-specific symptoms (Bernard, 1986). Behavioral manifestations may include apprehension, anxiety, irritability, and insomnia. Following the prodromal period, patients develop a rapidly progressive neurologic course, with a range of symptoms that may include disorientation, hallucinations, paralysis, nuchal rigidity, aerophobia, pharyngeal spasms, hydrophobia, hypersalivation, dysphagia, focal or generalized seizures, cardiac and respiratory arrhythmias, and hypertension, leading to coma and death (Matyas et al., 1999). A review of 32 human rabies deaths in the United States from 1980 to 1996 (Noah et al., 1998) identified agitation and confusion, hypersalivation, hydrophobia or aerophobia, limb pain, and weakness as the most commonly observed signs of clinical rabies. The cases had a median clinical period of 19 days (range, 7 to 28 days). In 12 of the 32 cases, the disease was only diagnosed postmortem. In the absence of intensive care and secondary support therapies, death usually comes in human rabies cases within 7 to 14 days of onset of symptoms, generally from respiratory failure (Gode et al., 1976; Jackson, 2007). In patients receiving intensive care, the disease eventually severely affects nearly every major organ system, and death occurs as a result of the cessation of cerebral and cardiovascular activity (Fishbein, 1991). There have been just six well-documented human survivals from clinical rabies worldwide, with outcomes ranging from full recovery without sequelae, or with some partial paralysis, to major residual neurologic impairment. Five of the six patients had a history of some pre- or postexposure vaccination. The sixth case was a teenager in Wisconsin in 2004 with a history of a bat bite on a finger 1 month prior to onset. This patient was maintained in a drug-induced coma and given antiviral agents. Her treatment included administration of ketamine, midazolam, phenobarbital, ribavirin, and amantadine. She improved and was released from the hospital with some neurologic sequelae but has experienced progressive neurologic improvement (Willoughby et al., 2005). The role of this strategy and therapeutic regimen in this survival has not been clearly established (Jackson, 2005). A possible role of an attenuated bat rabies viral variant has been proposed, as the virus was not isolated due to the early appearance of antibody and the lack of opportunity to examine postmortem CNS samples due to the favorable outcome. The application of similar treatment has not been successful in four subsequent cases with fatal outcomes (Hemachudha et al., 2006).

Rabies in vector animal species is similar in most aspects to the disease in humans, but the prodromal period often is followed by a stage of excitation with or without aggression either followed by or intermixed with periods of lethargy and depression. Progressive paralysis, which often begins referable to the site of exposure, may first be recognized in the posterior limbs or larynx. Paralysis of the throat may result in uncharacteristic vocalizations and accumulation of copious and stringy saliva from the mouth. During the excitative period, animals may exhibit self-mutilation as well as heightened and inappropriate sexual behaviors. The clinical presentations of rabies in animals have often been characterized as either "furious" or "dumb," with the prior involving substantial agitation and aggression and the latter predominantly involving lethargy and paralysis. In some cases, the animal may manifest both forms at different times in the clinical course (Hanlon, 2007). Livestock also may demonstrate aggressive and heightened sexual behavior, but facial and pharyngeal paralysis, hypersalivation, bellowing, straining, and posterior paralysis, leading to a "dog sitting" posture and then recumbency, are more common (Debbie and Trimarchi, 1992).

EPIZOOTIOLOGY OF ANIMAL RABIES

Rabies is maintained in cycles in bats, wild terrestrial carnivores, and domestic canine populations that serve as reservoirs and vectors of the disease. Specific variants of the virus are associated with each geographically and temporally defined wildlife cycle. The vectors are highly susceptible to the variant that has adapted to that population and are capable of transmission to conspecifics because of coincident aggressive behavior and infectious virus titers in their saliva. A sustained outbreak also is dependent upon an adequate vector population density and a host natural history that provides adequate opportunity for interspecific interactions within the characteristic clinical period. Rabies distribution in animals can be discussed in three general categories: domestic canine rabies, terrestrial wildlife rabies, and rabies in bats.

Rabies virus maintained in domestic dog populations accounts for 95% of all animal rabies cases reported globally (Fekadu, 1991) and still accounts for most of the zoonotic impact of the disease, with 90% of the human exposures to rabies and 99% of the human rabies deaths worldwide attributed to this cycle (Smith and Seidel, 1993). Although the development of potent vaccines in combination with stray dog control programs has been proven effective in extinguishing dog rabies epizootics, dog rabies is still epizootic in most countries of Asia and Africa (Knobel et al., 2005) and some areas in South America (Belotto et al., 2005). Worldwide, there are an estimated 60,000 human deaths each year

from dog-transmitted rabies, and 10,000,000 postexposure vaccination regimens administered as a consequence of this problem. In 1996, dog rabies was still a threat in 87 countries, with an estimated 2.4 billion humans at risk (WHO, 1997). Europe and North America controlled rabies in domestic dogs by stray animal control and widespread vaccination programs during the 1950s and 1960s, so it now accounts for less than 5% of animal rabies cases in those regions. Canine rabies is often associated with urban rabies cycles. Molecular analyses have shed light on the origins and distribution of canine rabies cycles seen throughout the world. The most geographically widely distributed group of rabies viruses is referred to as the "cosmopolitan" lineage (Smith and Seidel, 1993). Molecular epidemiologic studies, reviewed by Nadin-Davis and Bingham (2004), demonstrate that the genetic similarity of dog rabies variants (and some rabies viruses associated with wildlife rabies cycles) indicates that the virus variant was widely distributed during colonial times and that dogs of 14th-century Europe served as the source of these canine enzootics.

Rabies in terrestrial wildlife is also present nearly globally. The disease in red foxes has been prevalent in subarctic and northern parts of North America, in subarctic Asia, and in central and Eastern Europe. The raccoon dog and the gray wolf are reservoirs in north Eurasia (Wandeler et al., 1994). In Africa, the dog rabies variant has established enzootic rabies in wolf, jackal, and wild dog populations. In South Africa, a distinct strain of rabies is maintained in the yellow mongoose (Cleveland, 1998; Nel et al., 2005). Surveillance and reporting in Asia is sporadic, but the disease is present in foxes, jackals, and wolves (Kaplan, 1985). Rabies transmitted by dogs is reportedly a leading cause of death by infectious diseases in China, and previously unrecognized wildlife vectors are apparently important in rabies-related human mortality there (Zenyu et al., 2007). In South America, distinct rabies variants exist in some terrestrial wildlife (Belotto et al., 2005), but less is known regarding these because of the importance there of dog rabies (Krebs et al., 1995). Rabies is present in the Indian mongoose in Granada, Puerto Rico, Cuba, and The Dominican Republic of the Caribbean (Everard and Everard, 1985).

In North America, rabies in terrestrial species is also maintained in geographically defined outbreaks with a single antigenically or genetically distinctive variant. The variant is maintained by intraspecific disease transmission within the population of a single predominant vector species, after which the outbreak is named. Infection of other species in the area occurs, but these "spillover" cases have only rarely established sustained intraspecific transmission in another species. The disease may persist in the vector population for decades, and the geographic area affected can grow, diminish, or shift gradually or rapidly (Smith, 1996). The establishment of a rabies epizootic in a new area in a susceptible population can also be the result of human translocation of wildlife, exemplified by the introduction of raccoon rabies into the mid-Atlantic states in 1977 (Jenkins et al., 1998).

Raccoon rabies was first recognized in Florida in the 1940s and became endemic in the Southeast. This intense outbreak presently affects areas of the 20 eastern states from Florida to Maine and three eastern provinces of Canada (Blanton et al., 2007). The epizootic has been characterized by a rapid northeasterly spread, very large numbers of rabid raccoons in newly affected areas, and a subsequent cyclic nature, without ever completely dissipating in areas once affected. Spillover from this outbreak has occurred to an exceptional diversity of other mammalian domestic and wild species, most commonly, by far, to striped skunks. However, only a single human rabies case from this variant has been identified (CDC, 2003).

Skunk-vectored rabies enzootics exist across a broad region of the continent, including most of the central states and provinces and California. The viruses associated with this outbreak are actually composed of three genetically distinct lineages, maintained in the north central, south central, and Pacific coast areas (Orciari, 1995). Where rabies exists in other North American terrestrial vectors, there is significant spillover into skunks. The skunk cases in those areas result from infection with the outbreak-associated variant, and the distribution in skunks is limited to those areas where the disease exists in the primary vector (Guerra et al., 2003).

Rabies exists in red foxes in Alaska and the Canadian provinces of Ontario and Quebec. The variant is similar in each region, as these outbreaks are vestiges of the southward spread of Arctic fox rabies that became established in the red fox and swept across northern areas of the continent during the 1950s (MacInnes, 1988). Two different variants of rabies virus persist in gray fox populations of west Texas and in Arizona (Smith, 1989).

A relatively recent emergence of dog- and coyote-vectored rabies occurred in southernmost Texas, adjacent to a long-standing focus of canine rabies at the Texas-Mexico border. Beginning in 1988, the outbreak expanded northward to encompass most of south Texas. By 1999, oral rabies vaccination efforts had nearly extinguished this outbreak. With the last case of canine rabies occurring along the Mexican border in March of 2004, the CDC reported that the United States was canine strain rabies free in September 2007, after more than 2 years with no confirmed indigenously acquired cases of that variant (Blanton et al., 2007).

Spillover into other terrestrial mammal species exists in each of these areas of rabies endemicity, particularly to skunks and foxes. Sporadic cases are also seen in these areas in groundhogs, bobcats, coyotes, beavers, otters, opossums, deer, and rabbits. Spillover also occurs to unvaccinated domestic animals, including cats, dogs, ferrets, cattle, horses, sheep, goats, and swine. Rabies in large rodents, particularly woodchucks and beavers, is reported in areas of terrestrial rabies, especially in the midst of the raccoon rabies outbreak. Rabies is uncommon in other rodents and lagomorphs but has been occasionally confirmed in squirrels, rats, domestic rabbits, and Guinea pigs and even more rarely in prairie dogs, porcupines, chipmunks, and mice (Childs et al., 1997; Eidson et al., 2005).

Rabies in bats and rabies in terrestrial mammals are largely independent cycles. The rabies variants isolated from bats are antigenically and genetically distinct from those associated with terrestrial rabies (Smith, 1989). In bats, classical rabies virus infections (genotype 1 lyssavirus) are limited in distribution to the Western Hemisphere, where these mammals are of major importance as reservoirs and transmitters of rabies infection. Vampire bats are found throughout the more tropical areas of Latin America, and cattle losses from vampire bat-transmitted rabies range from 30,000 annually in Mexico alone (Brass, 1994) to more than 1 million in all of Latin America (Fenner et al., 1993). Human mortality from vampire bat-transmitted rabies continues to be reported, with 62 deaths reported from exposure to hematophagous bats in Latin America during the 10-year

period from 1993 to 2002 (Belotto et al., 2005), with intense local outbreaks occurring in some rural areas causing significant human mortality (da Rosa et al., 2006).

Rabies is widespread in insectivorous bats of North America, both geographically and by species. The disease has been confirmed in 49 states and in most of the 39 indigenous United States bat species. In 2006, the 1,692 laboratory-confirmed rabid bats from 49 states accounted for 24.4% of all United States animal rabies cases for the year. In 2006, six states reported rabies in bats but not in terrestrial species (Blanton et al., 2007). Most laboratory-confirmed rabid bats in the nation are not identified to species, but among those that are, most cases occur in the big brown bat, Mexican freetailed bat, and the little brown bat, all widely distributed common commensal species. Like rabies in terrestrial mammals, virus transmission in bats is mainly intraspecific, and distinct rabies variants can be identified antigenically or genetically for specific bat species (Smith, 1996). Although the analysis of bat rabies variants is hampered by a failure to identify the species of most bats tested and submission biases that result in most bats that are tested being of the common commensal species, associations of particular variants with the major colonial and migratory species have been described (Smith et al., 1995; Nadin-Davis, 2007). Occasional interspecific transmission of these variants is recognized in bats and, less frequently, isolated transmissions to terrestrial mammals. Although terrestrial mammals infected with bat variants can have virus in their salivary glands during clinical disease (Trimarchi et al., 1986), prior to 2001 there had been no direct observation that infection with a bat variant has established an outbreak in a terrestrial species. In 2001, an outbreak of rabies in skunks in Flagstaff, AZ, emerged that was identified as the consequence of infection with a big brown bat variant of rabies virus (Leslie et al., 2006). Skunk population control and vaccination programs targeting this outbreak were conducted in subsequent years, and no cases associated with this variant were reported in skunks in the area in 2006 (Blanton et al., 2007).

While estimates of disease prevalence in samples from random collection of asymptomatic bats at roosts are generally less than 1%, rabies positivity rates among bats submitted to public health laboratories for testing range from 5 to 15% (Brass, 1994). In New York State from 1987 to 2006, 3.2% (1,636 of 51,170) of bats submitted for public health testing were found to be rabid (C. V. Trimarchi and R. J. Rudd, unpublished data). There is an obvious strong bias toward sick and injured bats in these public health samples. Nevertheless, these rates are relevant for public health decisions because they accurately reflect the likelihood of rabies infection in individual bats encountered under common circumstances by people and pets.

The closely related European bat lyssaviruses 1 and 2 (genotype 5 and 6 lyssaviruses), the Australian bat lyssavirus (genotype 7 lyssavirus), and the less closely related Lagos bat virus (genotype 2 lyssavirus) and Duvenhage virus (genotype 4 lyssavirus) of Africa broaden the distribution of rabies-related encephalitis associated with bats. There are four lyssaviruses recently identified in Eurasian bats that are candidates to become additional genotypes (Hanlon, 2005). Current information on the prevalence, vectors, and distribution of the non-genotype 1 lyssaviruses is based on limited surveillance to date. These viruses are capable of producing rabies-like encephalitis in humans or other mammals and may eventually prove to be of greater epidemiologic importance as we learn more about their natural history. Molecular epidemiologic studies support the hypothesis that lyssaviruses existed in bats (chiropterans) long before they were present in terrestrial carnivores, that ancient spillover events resulted in the North American raccoon rabies variant and cycle, and that another event gave rise to the cosmopolitan canine rabies lineage (Badrane and Tordo, 2001). A molecular clock model suggests that the time of the most recent common ancestor for current bat rabies virus variants in the Americas was the mid-1600s (Hughes et al., 2005).

EPIDEMIOLOGY OF HUMAN RABIES

Worldwide, data regarding human rabies are largely unreliable. Underestimates are common because human rabies in developing areas is largely a rural problem, and many rural cases go undiagnosed or unreported (Nicholson, 1994). Human rabies is still common in developing, tropical countries of Africa, Asia, and Latin America, with an estimated 35,000 to 60,000 annual deaths worldwide. Because most of these deaths are due to rabid dog bites and a lack of or inadequate rabies postexposure prophylaxis (PEP), the mortality from the disease is largely a problem of poverty and inaccessibility to health care (Meslin et al., 1994).

In the United States, human rabies deaths became uncommon following control of dog-vectored rabies in the early 1950s. There were 99 cases during the 1950s, 15 in the 1960s, 23 in the 1970s, 10 in the 1980s (Noah et al., 1998), 27 in the 1990s, and 24 from 2000 to 2006. Of 51 human rabies cases from 1990 though 2006, 10 of the cases were acquired by dog bites occurring outside the United States. Of the 41 cases in which the infection was acquired in the United States and Puerto Rico, two were of a variant associated with indigenous domestic dogs, one was of a variant associated with raccoon rabies, one was of a variant associated with mongoose and dog rabies in Puerto Rico, and 37 were of a variant associated with insectivorous bats (Blanton et al., 2007).

LABORATORY DIAGNOSIS

The rabies laboratory has long played a prominent role in rabies control. The laboratory diagnosis of rabies is most often performed for the postmortem examination of animals that have potentially exposed a human to the disease by a bite or other transdermal contact with saliva or neural tissue. Because the modern rabies laboratory can provide reliable results on the day of receipt of the specimen, the physician's decision to provide or withhold rabies treatment following a bite from a suspect animal is commonly based upon the laboratory tests performed on the animal's brain. It is this function that dictates the uniquely high standards of sensitivity and specificity required of these tests, as a false-negative result easily could have the consequence of human mortality. Alternatively, if a delay in testing for rabies is unavoidable, it may be appropriate to initiate rabies treatment. Subsequent negative results from a reliable laboratory would justify terminating PET.

Examination for evidence of rabies infection is also performed on samples from rabies-suspect animals that have potentially exposed domestic animals and in the differential diagnosis of encephalitis in domestic animals, even in the absence of human or animal exposure. The rabies laboratory supports surveillance for the disease in wildlife to aid in the proper allocation and targeting of rabies control programs, such as the efforts to vaccinate wildlife with oral baits.

The combined data generated by examination for all reasons are used to define epizootiologic patterns useful for animal bite management when the offending animal is not available for observation or testing.

Not all biting animals need to be killed and tested. Because of laboratory data and empirical knowledge of virus shedding periods prior to onset of signs of the disease, dogs, cats, and ferrets can be confined and observed for signs of rabies for 10 days following a bite to rule out rabies transmission (see "Human Rabies Prevention" below). This sometimes is extended to other domestic species on a case-by-case basis.

Wild animals, especially bats, foxes, skunks, and raccoons, that have bitten or otherwise potentially exposed a human, should be tested immediately. Because rabies is a disease affecting mammals, it is never necessary to test arthropods, amphibians, reptiles, or birds. In the United States, small rodents, including mice, rats, and squirrels are essentially free of rabies, and therefore routine examination is not required; exceptions include rodents involved in unprovoked attacks in areas where rabies is endemic and larger rodents such as woodchucks, muskrats, and beavers (Childs et al., 1997).

Diagnosis of Rabies in Animals

Rabies diagnosis can be achieved with 100% sensitivity only by the postmortem examination of brain tissue. Throughout most of the prolonged incubation period of rabies there is no reliable means to rule out infection—there is no rise in circulating antibody titer, and neither rabies virus, its antigens, nor rabies RNA can be reliably identified. This is due to the limited and unpredictable distribution of virus and its proteins during the retrograde axoplasmal movement from the site of the exposure to the CNS during rabies pathogenesis. Rabies virus, its antigens, and rabies RNA do not move centrifugally away from the CNS during the incubation period (Charlton, 1988). Also, testing of samples of saliva collected antemortem from animals cannot be used with certainty to exclude the possibility of rabies infection and transmission by bites from the animal because virus may be present intermittently in saliva in infected animals (Rupprecht et al., 2002). However, modern methods can always identify the presence of rabies virus in the brain of a rabid animal that dies or is euthanized during, or up to several days before, the onset of the clinical signs of the disease. Therefore, after an exposure, when a decision is made to sacrifice and test the animal for rabies infection, it is never necessary to delay testing for further development of the disease to achieve a reliable diagnosis. Most importantly, centrifugal spread of the virus from the CNS to the salivary glands (and therefore a potentially infectious bite) does not precede the appearance of demonstrable rabies antigen in the brain (Charlton, 1988). Therefore, a negative result of brain examination by acceptable methods ensures that the bite of the animal could not have caused an exposure to rabies.

Collecting, Preparing, and Submitting Rabies Specimens

The animal species, nature of the exposure, variant of rabies virus, and time and cause of death may affect the terminal distribution of rabies virus and its antigens in the brain. Generally, the brain stem, the cerebellum, and the hippocampi constitute the best diagnostic samples, and areas of each tissue are examined to provide a reliable negative report. Therefore, the intact head is the preferred diagnostic sample for the postmortem diagnosis of rabies in animals. An animal can be euthanized for rabies examination with barbiturate or nonbarbiturate injectables or gases or by other humane means that do not damage the brain. Immediately preserve the specimen by cooling to 4°C, maintaining refrigeration until it arrives at the laboratory. Should refrigeration not be possible, freezing is an acceptable but less desirable alternative. A single freezing and thawing will not prevent reliable diagnosis, but freezing will make the dissection more difficult and may delay the test. Repeated freeze-thaw cycles can damage the specimen. The head should be removed from the neck before the first vertebrae, with caution to avoid contaminated injury, contamination of mucous membranes, or creation of infectious aerosols. Those capturing suspected rabid animals or handling and decapitating the carcass should receive appropriate training and rabies preexposure immunization (CDC, 2008). Submit bats and other very small animals intact to avoid damage to the animal's CNS during decapitation. For large livestock species, including cattle and equines, sample portions of the brainstem and cerebellum can be removed through the foramen magnum by a veterinarian (Debbie and Trimarchi, 1992).

The specimen should be directly and immediately transported to the laboratory. Specimens can also be shipped by a prompt parcel delivery service if properly packaged and labeled. Some state laboratories provide standard rabies specimen containers for shipping heads to the laboratory. Current federal guidelines applicable to the required packaging of potentially infectious samples, to which shippers must strictly adhere, can be found at the U.S. Department of Transportation website at www.dot.gov. An envelope attached to the outside of the container should contain a fully completed standard rabies specimen history form, if available. If no form is available, provide all the significant information including the names, addresses, and telephone numbers of the owner, complainant, and all humans and animals in contact as well as information on the clinical observations, date of death or means of euthanasia, exact location of capture, and information on the person or agency to receive the report. Generally, reports of rabies-positive specimens are made immediately by telephone. Reporting practices vary widely, however, and the submitter should ascertain local practice by contacting the local, regional, or state health department.

Dissection and Sample Preparation

When the entire head is received at the laboratory for examination, the flesh is removed from the cranium and an anterior and two lateral cuts are made in the cranium by chisel or saw to permit the calvarium to be reflected posteriorly. After removal of the meninges, the cerebellum and the brainstem are removed. An alternative method, useful for surveillance-only examinations, employs the removal of a core of brain tissue by the insertion of a soda straw or similar hollow tube into the foramen magnum and advancing forward to capture samples of the brainstem, cerebellum, and hippocampus that can then be forced out of the straw and used for slide and suspension preparation (Barrat, 1996). Touch impressions or slip smears of each tissue are made on microscope slides immediately. For some enhanced surveillance surveys, using a rapid immunohistochemical screening method described later, a cross-section of brainstem alone is utilized. Mainly used for research purposes and for examination of nonneural tissues, frozen sections of tissue can be prepared using a cryostat. A 10% suspension of a mixture of the diagnostic tissues is also made in suitable diluents for animal inoculation or cell culture virus

isolation. A sample of each brain tissue is saved at –70°C for further testing.

Direct Immunofluorescence for Rabies Antigen

With an achievable sensitivity and specificity approaching 100% and a routine turnaround time of less than a day, microscopic examination of brain tissue stained with the direct immunofluorescence assay (DFA) is the preferred procedure for the diagnosis of rabies. Among the findings of a National Working Group on Rabies Prevention and Control was the need for a minimum national standard for the laboratory diagnosis of rabies (Hanlon et al., 1999). In response to this recommendation, a committee was formed from representatives of national and state public health laboratories to evaluate the procedures employed by rabies diagnostic laboratories in the United States. Both the National Working Group and this committee have as their goal uniformity and the improvement of the overall quality of rabies testing through the formulation of guidelines and standards for equipment, reagents, training, laboratory protocols, quality assurance, and laboratory policy for rabies diagnosis. As a first step to attaining this outcome, the committee prepared a standardized protocol for the analytical phase of rabies testing using the DFA and evaluated the protocol by comparison testing of 435 samples submitted to public health laboratories for rabies diagnosis. The standardized protocol was developed from published procedures and the collective laboratory experience of the committee members. The group recognized that a range of possible methods may achieve the desired outcome for some of the less critical steps in the diagnosis of rabies and that laboratory policy may be defined regionally in some cases. However, the goal of the group was to establish a single protocol by which all other methods could be validated by comparison. Furthermore, uniformity of procedures in the national laboratories performing these examinations permits the elucidation of diagnostic problems and solutions without the obscurance provided by multiple variables. The recommendations included in this document should be closely followed to ensure a test of highest sensitivity and specificity. Modifications or shortcuts in procedures could lead to false-positive or false-negative results and nonspecific reactions (Rudd et al., 2005). The new standard protocol requires the routine use of two different diagnostic conjugates on each test, confirmatory protocols for detection of minimal antigen distribution, and adequate sampling of brain tissues. The protocol has been placed on the websites maintained by the Centers for Disease Control and Prevention (http://www.cdc.gov/ncidod/dvrd/rabies/professional/publications/DFA_diagnosis/DFA_protocol-b.htm).

Since the development of the first human rabies vaccines, accurate and timely diagnosis of rabies infections in animals has been essential to the prompt and successful postexposure prophylaxis of humans. In addition, accurate diagnosis is essential to the maintenance of a disease surveillance system, which influences public health decisions determining animal quarantine, vaccination, control, and related allocation of government resources. Thus, the serious nature of this zoonotic disease mandates the appropriate use of diagnostic techniques to ensure the highest obtainable sensitivity and specificity. Adherence to the accepted standardized and validated protocol for the fluorescent antibody test in the postmortem diagnosis of rabies in animals is one essential step toward accomplishing that goal.

Glass microscope slides containing slip smears or impression slides of brain tissue, frozen sections of other tissues, or monolayers of tissue culture cells are fixed for 1 to 4 h in acetone at –20°C to ensure permeability of the cells to the diagnostic antibodies and to aid in tissue adherence to the slide. An alternative microwave fixation process has been described (Davis et al., 1998) but has not found widespread use. After air drying, the slides are flooded with the diagnostic immunofluorescence reagent. These reagents are made by the conjugation of fluorescein isothiocyanate (FITC) to purified immunoglobulin G (IgG) specific for rabies nucleocapsid protein. The antibodies can be extracted and purified from antiserum of rabies-hyperimmune hamsters, goats, rabbits, or equines. The development of mouse monoclonal antibody technology (Kohler and Milstein, 1975) has permitted production of highly specific and uniform diagnostic reagents. Employing a cocktail of these antibodies specific for different epitopes on the rabies antigen (Wiktor et al., 1980), a new generation of diagnostic conjugates has been developed for rabies diagnosis. The conjugated reagent may also contain a counterstain such as Evans blue (Rudd and Trimarchi, 1997). After a 30-min incubation at 37°C in a moist incubator, the diagnostic reagent is thoroughly washed off in multiple saline baths. The FITC-conjugated antibodies remain attached to the brain film only where rabies nucleocapsid protein is present. After air drying, coverslips are mounted using a low-glycerol-content mountant specifically designed for the rabies DFA (Rudd et al., 2005). Slides are examined using a light microscope fitted with a mercury vapor or xenon lamp and appropriate excitation and barrier filters to show FITC-labeled structures as yellow-green fluorescent objects against a dark background. Rabies-specific staining in brain tissue appears as characteristic round or oval intracytoplasmic inclusions most prominent in the large neurons of the cerebellum, hippocampus, and brainstem (Trimarchi and Debbie, 1991).

Rabies immunofluorescence testing on fresh brain tissue can be comparably sensitive or superior to isolation procedures (Rudd and Trimarchi, 1989; Webster and Casey, 1996). A true indication of the value and reliability of the DFA is demonstrated by the absence of any known human rabies cases in the United States when rabies PET was withheld based on an immunofluorescence-negative laboratory report during nearly 50 years of use. Important factors in the avoidance of false-negative results in rabies diagnosis include strict adherence to a uniform methodology, the use of proper scientific controls for all procedures, diagnostic conjugate quality and proper dilution, and optimization of lamp and microscope performance. The specificity of the procedure can also approach 100% agreement with virus isolation when procedures and practice ensure avoidance of cross contamination, and conjugate-diluent and wash salines and staining conditions are optimized to eliminate nonspecific fluorescence. The laboratory can use selected application of tests to confirm specificity of observed fluorescence, including paired staining with virus-absorbed and sham-absorbed conjugates and staining with FITC-labeled IgG conjugate specific for another antigen.

The diagnosis of rabies in animals is not a Clinical Laboratory Improvement Amendment 88 analyte. Therefore, proficiency testing is not mandatory. Voluntary rabies proficiency testing programs were conducted periodically from 1973 to 1992 and each year since 1994. The performance of rabies diagnostic laboratories enrolled in proficiency testing in the United States has been excellent when panels of rabies-positive and -negative test slides were evaluated by the DFA. Excellent consensus has been observed among participants for strong positive and negative test samples. Discrepancies

have mainly occurred with very weakly positive slides (Powell, 1997). One of the most important factors for efficacy of the rabies DFA is the laboratory's recruitment and retention of properly trained and experienced microscopists (Hanlon et al., 1999).

A critical requirement for sensitive rabies diagnosis by immunofluorescence is a good quality brain sample. Brain tissue exposed to chemical fixative, repeated freeze-thaw cycles, or elevated temperatures may result in denatured or masked rabies antigens, hampering recognition by the diagnostic reagents. Decomposition will affect the sensitivity of all rabies diagnostic procedures. Immunofluorescence tests may remain positive for a period after isolation of virus is no longer possible (Rudd and Trimarchi, 1989). Evidence of rabies infection by DFA on decomposed or mutilated tissue fragments may support a valid rabies-positive report, confirmable by isolation, immunohistochemistry, or molecular methods. One of the most difficult diagnostic decisions confronting the rabies laboratorian is to determine at what stage of decomposition it is no longer possible to issue a reliable negative rabies report. Certainly, once the CNS has become foul smelling, green in color, and with some liquefaction, negative results are not reliable (Rudd and Trimarchi, 1989). Occasionally, even specimens with the appearance of only early decomposition on gross inspection may result in slides with each microscopic field so overgrown with autofluorescing decomposition bacteria that reliable examination is not possible. Mutilation of the tissue by trauma to the extent that the necessary sample regions of cerebellum and brain stem are not available or unrecognizable also precludes a reliable negative result (Bingham and van der Merwe, 2002; Radlich, 2003). Generally it is not possible for lab personnel, based solely on a verbal description, to determine before the dissection whether or not a decomposed or mutilated specimen may be testable. Consequently, unless it is clear that the carcass is in the last stages of decomposition or is mutilated to the extent that no recognizable CNS exists, it is wise to submit compromised specimens to the laboratory for evaluation as to suitability.

Other Methods of Antigen Detection

Antigenic sites recognized by antinucleocapsid diagnostic reagents in the standard DFA may be masked by bonds created when CNS tissues are fixed by exposure to formalin. As a result, the sensitivity of the procedure is greatly reduced on fixed tissues. Advances have recently increased the sensitivity of direct and indirect immunofluorescence and immunohistochemical procedures applied to fixed tissue. Digestion of the tissue with enzymes such as proteinase prior to staining can expose the antigenic sites (Warner et al., 1997). Use of avidin-biotin complex amplification and high-affinity monoclonal antibodies for the first label has improved the performance of these procedures, so that they may be approaching the reliability of DFA on fresh tissues (Hamir et al., 1995; Whitfield et al., 2001). A direct rapid immunohistochemical test employing a short formalin fixation of fresh or glycerol-preserved brain impressions and requiring no specialized equipment has been demonstrated to be of utility for testing under field conditions or for countries with limited diagnostic resources (Lembo et al., 2006). Further evaluation is necessary before negative results on chemically fixed tissues can be used for public health decisions. When applicable, confirmatory testing of fixed tissues by another sensitive technique, such as reverse transcription (RT)-PCR, would be appropriate.

Rapid and simple diagnosis of some viral infections can be achieved using enzyme-linked immunosorbent assays (ELISA). A method has been developed to demonstrate the presence of rabies antigen, and recent methods utilizing avidin-biotin amplification show promise of improved sensitivity, approaching that of DFA and virus isolation (Bourhy and Perrin, 1996; Archana et al., 2002). They offer the advantages of automated reading and good reliability on partially decomposed tissues, offering field condition applicability. Performance on well-preserved specimens is poorer than other methods, however (Franka et al., 2004). While these techniques can be used as a backup to DFA examination and for epizootiologic surveillance testing, they are not widely used in U.S. public health laboratories. An avidin-biotin amplified dot blot enzyme immunoassay showed good sensitivity and specificity in one study on brain tissue of rabies-suspect animals but performed poorly on clinical samples, such as saliva, for antemortem diagnosis of human rabies (Madhusudana et al., 2004).

Histologic Examination

Negri bodies are intracytoplasmic, acidophilic inclusion bodies that can be demonstrated best in the Purkinje cells of the cerebellum and pyramidal cells of the hippocampus (Perl and Good, 1991) of many rabies-infected animals by microscopic examination of tissue stained with basic fuchsin and methylene blue (Tierkel and Antanasiu, 1996) or hematoxylin and eosin (Lepine and Atanasiu, 1996). The presence, distribution, and size of Negri bodies are related to the species of animal, variant of rabies virus, and duration of the clinical period before death or euthanasia. Demonstration of these pathognomonic inclusions is very specific and, coupled with evidence of encephalitic inflammatory response, may provide a reliable positive rabies diagnosis when reported by an experienced pathologist. However, the sensitivity of the method is poor, with numerous histologic surveys indicating that 25% or more of rabid animals have no demonstrable Negri bodies, severely limiting the value of this diagnostic method for medical decisions (Perl and Good, 1991).

Virus Isolation

The dire consequences of false-negative results in cases of human exposure support a continued practice of utilizing rabies virus isolation as a backup, confirmatory procedure, despite the proven sensitivity and reliability of the DFA. Virus isolation may be employed as a general quality control procedure or just applied to instances of bites to humans from highly suspect animals. In either case, it serves to sustain confidence in the reliability of the DFA results and to exonerate the microscopist of the full burden of responsibility. The propagation of virus in the laboratory is also a critical component for identification of virus variants and in production of diagnostic reagents. In vitro and in vivo methods are both widely employed.

The mouse inoculation test (MIT) was introduced for diagnostic purposes in 1935 (Webster and Dawson, 1935). It is a very sensitive and reproducible procedure. When used as a confirmatory procedure for DFA results, portions of the same tissues that are used for the microscopic examination are ground into a suspension, employing a diluent of physiologic salt solution containing serum and antibiotic supplements. The suspension is inoculated intracerebrally into weanling Swiss albino mice. The mice are carefully observed daily for 30 days for evidence of rabies infection. Mice that develop illness during the observation period are

immediately euthanized, and the brains examined by DFA. A valuable attribute of the MIT is its ability to detect small quantities of rabies virus even in very weakly positive specimens (Sureau et al., 1991). It can also be successfully applied to mutilated and decomposed samples (Rudd and Trimarchi, 1989). Its weakness, beyond the inherent environmental and ethical issues with the use of live animals in the laboratory, lies in the typical 7- to 20-day period between inoculation and recognized illness in the mice. The limitation of the procedure results from the possibility that if treatment were withheld following a bite due to a false-negative DFA, detection by this backup method would occur after a period that would cause great concern about vaccine failure (Rudd and Trimarchi, 1980). The period can be shortened by the use of neonates, with daily sacrifice and DFA examination of numerous individual animals, but this greatly increases the labor-intensive nature of the procedure and can be prohibitive in a laboratory performing routine diagnosis on large numbers of specimens.

The delay associated with the demonstration of virus by the MIT can be avoided by the isolation and identification of rabies virus on continuous cell culture (Rudd and Trimarchi, 1989). Tissue from the diagnostic regions of the brain of the suspect animal is ground into a suspension in a cell culture medium as a diluent. The suspension is incubated for one to several days after addition to cells of a continuous cell line selected for its susceptibility to infection with rabies virus, generally a mouse neuroblastoma cell line (Umoh and Blenden, 1983; Rudd and Trimarchi, 1987). The sensitivity of the procedure can be increased with the treatment of the cells with DEAE dextran (Kaplan et al., 1967). The test can be performed in tissue culture slides, 96-well plates, or Teflon-coated slides. The cell monolayers are then washed, fixed in cold acetone or a formalin-methanol mix, and examined by DFA. If infectious rabies virus is present in the brain tissue, characteristic intracytoplasmic inclusions of rabies antigen will be observed in fluorescent foci in the cells. The sensitivity of the procedure is comparable to that of the DFA and the MIT (Rudd and Trimarchi, 1989; Webster and Casey, 1996). Because results are available within a few days of or as soon as 18 h after (Bourhy et al., 1989) receipt of the specimen, it serves as a much better means of confirming negative DFA results, as a false-negative DFA would be recognized in a period of time permitting timely initiation of PET (Rudd and Trimarchi, 1980).

Molecular Methods

Diagnostic methods targeting the nucleic acids of rabies virus may use existing viral nucleic acids in samples or those resulting from an amplification process. Direct molecular methods for the diagnosis of rabies employ probes for the presence of existing rabies virus RNA in tissue samples using dot or blot hybridization assays (Ermine et al., 1988). These methods generally lack sufficient sensitivity to show the presence of rabies RNA among the total RNAs of the sample, except in the more heavily infected samples (Tordo et al., 1995), and are therefore not generally used for diagnosis. Applications of in situ methods for use on formalin-fixed tissues to discriminate viral strains (Nadin-Davis et al., 2003) and as a possible confirmatory test for results of DFA examination of fixed samples (Warner et al., 1997) have been described.

Amplification of viral nucleic acids with RT-PCR facilitates more sensitive and useful techniques (Saiki, 1989). Early methods for lyssavirus amplification, detection, and characterization were described by Sacramento et al. (1991) and updated for application with commercially available reagents by Tordo et al. (1995) and Nadin-Davis (1998). A very thorough and detailed discussion of current methods is provided by Trimarchi and Nadin-Davis (2007). RNA extracted directly from infected brain material by commercially available reagent kits is reverse transcribed to cDNA, which is then amplified by PCR. RT-PCR requires primers, or short synthesized oligonucleotide sequences, derived from conserved regions of the viral genome to permit amplification of all members of the lyssavirus genus. The contribution by Nadin-Davis (2007) in *Rabies* provides a very detailed discussion and listing of primers in use. Because the N gene is the most highly conserved region of the lyssavirus genome (Le Mercier et al., 1997), to provide a broadly reactive diagnostic test, the nucleoprotein or N protein gene is targeted. This also allows for direct comparison between genetic characterization and that using monoclonal antibody panels that target nucleoprotein. The optimal sensitivity is achieved with a nested PCR, in which a second round of amplification is performed on the initial RT-PCR product using primers internal to the original primers (Kamolvarin et al., 1993). It may be that nested PCRs are necessary due to inefficiencies in first-round primer mismatches. It has been suggested that well-matched primers for the first-round PCR in most cases can eliminate the need for a nested method (Trimarchi and Smith, 2002).

The products of PCR amplification of the rabies genome can be detected and analyzed in numerous ways, including direct visualization in agarose gels stained with ethidium bromide following electrophoresis. Indirect detection can be done using DNA probes revealed by radioactive labels, digoxigenin immunologic detection, or enzymatic revelation with indicators such as alkaline phosphatase.

Molecular methods have important applications in the rabies laboratory: (i) in the antemortem diagnosis of human rabies (Noah et al., 1998); (ii) as a sensitive backup test to DFA; and (iii) when paired with restriction fragment length polymorphism or with nucleotide sequencing analysis in the epizootiologic investigations of rabies (Smith et al., 1991; Nadin-Davis et al., 1996). However, their use is limited in the routine diagnosis of rabies for the postmortem examination of animals following exposures to humans or domestic animals. For a number of reasons, DFA is unlikely to be replaced soon by RT-PCR for these examinations, and DFA remains the gold standard (WHO, 2005). The DFA for rabies antigen in brain tissue is rapid, sensitive, specific, easy to perform, and relatively inexpensive. Comparisons of sensitivity and specificity of DFA to virus isolation approach 100% agreement (Smith, 1999). In the United States, no human case of rabies has ever been attributed to contact with an animal found negative for rabies by DFA on brain material. Molecular assays are more labor-intensive, more time-consuming, and costlier than DFA.

Primer selection and the identification of truly universal primer sets for rabies and rabies-related viruses may still be a barrier to withholding rabies treatment based solely on negative PCR results. Generally, genomic nucleic acid variability is significantly greater in lyssaviruses than at the protein level due to genetic code redundancy (Bourhy et al., 1993; Kuzmin et al., 2005). Therefore, a false-negative result is potentially a bigger problem with hybridization of a short segment of nucleic acid to the RNA target than with an antigen-antibody bonding method such as the DFA.

Furthermore, while nested PCR can be 100 to 1,000 times more sensitive than DFA, this extreme sensitivity brings with it a need for extraordinary quality control practices to

avoid false-positive results. Small fragments of RNA generated by tissue processing during necropsy, or transferred from sample to sample during RNA extractions, could generate false positives during the 100,000 or more public health rabies examinations conducted annually in laboratories in the United States. Great attention must be focused upon operational requirements for molecular assays, without which false-positive results may occur (Kwok and Higuchi, 1989). Design of facilities, well-planned testing algorithms, and strict adherence to molecular workflow practices that preclude the movement of materials or personnel from "dirty" postamplification areas to upstream "clean" areas are essential (Cooper and Poinar, 2000). Use of mock extraction controls, either water or, preferably, known rabies-negative brain tissue, is essential to control for cross contamination. Positive and negative samples should be included on each assay to confirm the success of each test run. An internal control for template integrity is required to evaluate a sample's suitability for PCR, which can be deleteriously affected by degradation by environmental factors or the presence of PCR inhibitors.

But molecular methods have well-recognized advantages over antigenic assays for some sample types and conditions. Several studies (Heaton et al., 1997; Whitby et al., 1997; David et al., 2002) have demonstrated that a molecular assay can be greatly more sensitive than DFA when applied to brain tissue in advanced stages of decomposition: a condition that is not rare in specimens received for public health testing. Some sample types, including saliva and cerebrospinal fluid (CSF), are not conducive to DFA's microscopic examination, for example, in the antemortem diagnosis of human rabies. Molecular methods may find more common application as a confirmatory test in the common practice of requiring a confirmatory assay in instances of negative DFA results on specimens involved in a potential human exposure. For this purpose, molecular methods have advantages of a shorter completion time and sometimes, greater sensitivity (Picard-Meyer et al., 2004).

Real-time PCR assays, in which products of amplification are identified by specific, labeled probes as they are produced, permit improvements in turnaround time, allow for quantitative analysis, and can reduce the potential for cross contamination by eliminating the need to open vials to permit analysis of the products of amplification. A number of applications of these methods to lyssavirus detection have been described (Black et al., 2002; Hughes et al., 2004; Wakely et al., 2005). Despite the potential advantages of the approach, use in postmortem detection of rabies in animals for public health purposes is not yet feasible due to a demonstrated need to employ many sets of primers and probes to detect the wide range of rabies virus variants that might be encountered in North America. Hughes et al. (2004) concluded that mismatches between primer and probe sequences and target sequence are sufficiently detrimental to amplification and product signal, and microdiversity within clades of rabies viruses is sufficiently common to reduce the reliability of the methods for the detection of the wide range of rabies virus variants in the United States. A nucleic acid sequence-based amplification method has been used successfully for the detection of rabies in antemortem samples of saliva and CSF from human patients (Hemachudha and Wacharapluesadee, 2004).

Diagnosis of Human Rabies

Despite the dire prognosis in rabies infection, testing should be done in all cases of acute, progressive human encephalitis of unknown etiology, even in the absence of a history of bite exposure (CDC, 1997). As a result of the efficacy of modern PET regimens, human rabies cases in the United States and other developed nations are no longer commonly associated with vaccine failure. Also in these regions, rabies prophylaxis is provided in all cases of known exposures and even in most cases of suspected exposure to rabies. Therefore, human cases are most often identified in the absence of a clear history of suspicious animal bite or other exposure. In numerous recent human rabies cases in the United States, the disease was not suspected or diagnosed during the clinical illness and was only recognized postmortem and sometimes after a lengthy delay (Noah et al., 1998).

The possibility of a successful outcome from a novel therapeutic approach to a human rabies infection was demonstrated in the recovery following treatment with induced coma and antiviral drugs of a Wisconsin teenager in 2004 (see discussion in "Clinical Rabies" above). It is likely that delayed diagnosis would reduce the efficacy of such treatments (Willoughby et al., 2005). The reports of human-to-human rabies transmission as a result of organ transplantation further emphasize the potential value of early intravitam detection of rabies infection (Trimarchi and Nadin-Davis, 2007). Antemortem diagnosis is also a valuable tool to permit early identification and PET of family and health care staff potentially exposed by contact with the patient's saliva. It also aids in patient management and allows efforts to prepare the family for the likely fatal outcome of the disease. Postmortem diagnosis of rabies in cases of fatal encephalitis of unknown etiology is critical to gain greater knowledge of the prevalence of rabies encephalitis in humans, the frequency of failure of pre- and postexposure vaccination, and the probable vectors and variants that pose the greatest risk to human health. The recognition of each human rabies case is very important in identification of highest risk exposure routes, vectors, and rabies virus variants—information essential to the development of effective rabies control and exposure management protocols.

Although brain biopsy would be the most sensitive antemortem diagnostic method, the risks associated with the procedure make its use uncommon. There are numerous less invasive intravitam tests that can confirm rabies infection. However, rabies virus, its antigens, and rabies RNA do not move centrifugally away from the CNS during the incubation period and only slowly during the clinical period (Charlton, 1988). Similarly, humoral antibody responses do not occur during the incubation period and neutralizing antibodies are frequently not demonstrable until the second week of illness. Antemortem diagnosis is therefore attempted by the analysis of numerous tissues by several methods searching for rabies-specific antibody in serum or CSF or viral antigen, live virus, or viral RNA in body fluids (saliva), peripheral nerves (skin biopsy), or epithelial cells (corneal impression). Antigen detection can best be accomplished by DFA performed on a full thickness skin biopsy specimen taken from the nape of the neck and including several hair follicles (Smith, 1999). DFA also can be used to demonstrate rabies antigen in corneal impression slides (Zaidman and Billingsly, 1997). It is recommended that these samples be taken by an ophthalmologist because of the risk of corneal abrasions. Virus isolation by MIT or cell culture inoculation can be applied to saliva and CSF. Antibody assay for this purpose can be performed by neutralization test, ELISA, or indirect DFA on serum and CSF. Nested RT-PCR is applied to saliva, skin biopsy specimens, corneal impressions, or CSF. Demonstration of rabies antigen by

DFA in any solid tissue, of rabies RNA in saliva, rabies antibody in serum and CSF, or isolation of rabies virus from any tissue is confirmatory of rabies infection. Antibody in serum alone may not be indicative of clinical rabies in a patient with known or unclear history of rabies vaccination or very recent treatment with rabies immune globulin (RIG).

The samples for antibody assay are 1 ml or more of CSF and serum, submitted in a plastic tube or vial. The skin biopsy specimen can be submitted on a gauze sponge moistened with sterile physiologic saline and sealed in a small plastic container. Corneal impression slides should be submitted in a plastic slide container with the surface of the slide containing the impression clearly marked. A 1-ml sample of frank saliva should be collected in a plastic sputum jar. Alternatively, a buccal swab can be taken and submitted immersed in a tube containing 1 ml of sterile saline. All of these samples can be stored at –70°C and shipped on dry ice. Postmortem testing methods for human rabies are similar to those described for animals. If the patient dies and an autopsy is performed, the ideal sample for postmortem diagnosis are 1-cm^3 samples of unfixed cerebellum and brain stem preserved by refrigeration. Samples must be shipped on dry ice packaged in compliance with applicable federal shipping guidelines for infectious agents. At the time of this writing, International Air Transport Association guidelines are followed that characterize rabies virus as a "biological substance, category B" in clinical specimens and as a "biological substance, category A" only when it has been cultured (International Air Transport Association, 2006).

The laboratory should be contacted immediately when human rabies is suspected. If original samples collected in the first week of symptoms do not disclose evidence of rabies infection, it must be understood that this does not conclusively rule out rabies and that repeat samples may be necessary because antemortem tests may remain negative well into the clinical period. Review of 32 human rabies deaths in the United States from 1980 to 1996 (Noah et al., 1998) disclosed that in 12 of the cases rabies was not suspected until after death. Of the remaining 20 cases, antemortem evidence of rabies was found by one or more tests in 18 cases. Antibody to rabies was detected in 10 of 18 patients tested, and virus was isolated from saliva in 9 of 15 cases. Rabies RNA was detected by RT-PCR in each of the 10 patients tested by this molecular method. However, nested PCR was required in almost all cases to detect the extremely small amounts of viral RNA in the samples collected antemortem. Antigen was demonstrated by DFA in skin specimens from 10 of 15 patients tested and in corneal impressions from 2 of 7 patients tested, and antigen was present in brain biopsy specimens from all three patients examined in that manner. In another report (Crepin et al., 1998), rabies sequences were detected in 4 of 9 saliva samples with a nonnested RT-PCR assay. Formalin-fixed brain tissue can be examined in suspected human cases in the same manner as indicated for animal tissues.

Antemortem Diagnosis of Animal Rabies

It is possible to apply the methods described for antemortem diagnosis of human rabies to suspect animals as well. Skin biopsy has been demonstrated to be particularly sensitive when applied to biopsy specimens taken from the snouts of terrestrial carnivores, which permits examination of the innervation of the tactile hairs (Blenden et al., 1983). However, the same limitations apply to antemortem diagnosis in animals: since all tests can remain negative well into or throughout the clinical period, negative results do not rule out rabies. Rabies cannot be reliably diagnosed by current methods during most of the incubation period. Claims that molecular testing of saliva of a biting animal can be used for decisions of bite management are false, as rabies virus may be shed in saliva sporadically prior to and during the clinical period (Charlton, 1988). Therefore, these tests are of little value for public health decisions and should never be a substitute when circumstances require 10-day observation or euthanasia and examination of brain tissue.

Rabies Virus Variant Typing

Methods that characterize the antigenic and genetic attributes of isolates of rabies virus and the rabies-related lyssaviruses now enable the laboratory to identify the virus variants responsible for epizootics as well as individual cases of rabies in animals and humans. Distinctive differences in the variants responsible for the major terrestrial outbreaks worldwide, the numerous bat rabies variants, the laboratory strains of rabies virus, and the rabies-related lyssaviruses are distinguishable by these means. Rabies variant identification yields a greater knowledge of the epizootiologic relationships of virus and vectors, allowing the development of more effective animal contact guidelines and rabies control strategies. Reaction patterns in indirect DFA, employing panels of monoclonal antibodies specific for unique viral nucleocapsid epitopes, permit such discrimination (Smith et al., 1986). The immunofluorescence assays can be performed on brain tissue of the original patient or mouse- or cell culture-passaged virus. Genetic analysis permits more precise detail of the evolutionary relatedness of isolates, investigation of the spatial and temporal changes that may occur, and particularly, the measure of similarity among virus isolates. This is accomplished by the extraction, transcription, and amplification of the RNA of an isolate by RT-PCR and subsequent sequence analysis of the cDNA nucleotide or amino acid sequence for the entire or partial nucleocapsid or glycoprotein genes (Tordo et al., 1992). Using computer algorithms to perform pairwise comparisons, estimates of genetic identity can be calculated and expressed as percent homology among isolates (Smith et al., 1992). Molecular epidemiologic studies by many investigators are reviewed thoroughly by Trimarchi and Nadin-Davis (2007), with description of the complex relationships among genotype 1 rabies viruses and the other lyssavirus genotypes.

Rabies Antibody Assay

Assays to demonstrate and quantify rabies antibody in serum from humans and animals serve numerous functions in rabies diagnosis and control. Serologic testing for rabies-specific antibody titer is performed on human serum to determine the response to pre- and postexposure vaccination and to determine the timing of booster vaccinations to maintain a rabies-immune status. Evidence of rabies antibody in serum and CSF is used in the antemortem diagnosis of rabies in humans. Antibody detection in human and animal vaccinees is used in vaccine efficacy trials and in evaluation of field wildlife vaccination campaigns. Long quarantines for cats and dogs entering rabies-free countries can be reduced if an immunologic response may be confirmed by this means (WHO, 1992).

Because these antibody tests are generally employed to measure immune status, the most widely used assays measure neutralizing antibodies. Constant-virus/varying-serum dilution neutralization assays are most commonly employed. Dilutions of heat-inactivated serum are combined with a

standardized amount of rabies virus and then incubated for 1 h at 37°C. Mouse or cell culture inoculation is performed after incubation to demonstrate residual virus after incubation. The mouse inoculation test developed in 1935 (Webster and Dawson, 1935) is a very reproducible method, still employed in some laboratories, and is used as the standard to evaluate other procedures.

The commonly used cell culture virus neutralization techniques are those in which residual virus is demonstrated by immunofluorescence in the inoculated cell monolayers. The most widely employed test for human postvaccinal titer determination is the rabies fluorescent focus inhibition test (RFFIT) (Smith et al., 1996). This technique determines the serum neutralization endpoint titer by a mathematical interpolation of the number of microscopic fields containing fluorescent foci at serum dilutions of 1:5 and 1:50. While results may be given as reciprocals of the calculated endpoint dilution, they are often expressed in terms of international units of neutralizing activity, determined on each test by the titration of a standard reference serum. Alternative in vitro methods that actually determine the last dilution of the patient's serum that neutralizes the virus challenge in a manner similar to the standard mouse test are also utilized and generally report titers in international units (Trimarchi et al., 1996). One of these tests, the fluorescent antibody virus neutralization, has been selected by the Office International des Epizootics as the standard for examination of animal sera for evidence of successful vaccination prior to movement into rabies-free areas (Office International des Epizooties, 1996). A recent comparison of the sensitivities of the fluorescent antibody virus neutralization and the RFFIT in one laboratory concluded they are comparable if quality control measures are stringent, particularly assurance that a well-defined challenge virus, obtained from the same source, was utilized (Briggs et al., 1998).

Other serologic tests are employed for rabies antibody assays, particularly for research and vaccine evaluation procedures. A high degree of sensitivity and specificity are reported with an ELISA directed against whole virus for the measurement of neutralizing antibodies (Savy and Atanasiu, 1978). The rabies G protein should compose the immunosorbent for the ELISA. A competitive ELISA, employing a neutralizing monoclonal antibody, reportedly achieves a high degree of sensitivity, the freedom from the need for species-specific intermediate antibodies, and measurement of neutralizing antibody (Elmgren and Wandeler, 1996). Because agreement between ELISA and neutralization tests is not always complete, ELISAs that are not FDA licensed should not be used to make human therapeutic decisions (Conti, 2001).

RABIES CONTROL

If the control of rabies is defined as elimination of human mortality from the disease, then it can be achieved by success with the following strategies, individually or in combination: elimination of the virus in animal populations, elimination of human exposure to infected animals, prevention of human infection by prior or postexposure vaccination, or development of an efficacious cure for clinical disease. Because rabies virus has demonstrated adaptability to such a wide variety of host populations, eradication of the virus, as has been achieved for smallpox, may not be a realistic goal. Control in domestic dog populations and some wildlife vectors in geographically defined areas has proven to be accomplishable by stray dog control coupled with vaccination programs and by oral rabies vaccination campaigns in wildlife (Slate et al., 2005). However, resource limitations, an increasing movement of domestic and wild animals, and a greater knowledge of the significant role and distribution of rabies and rabies-related viruses in bats threaten to make even regional elimination unlikely for most of the world. Modern cell culture vaccines and purified rabies immune globulins of human origin have made safe and highly efficacious pre- and postexposure immunization a reality, yet resource limitations prevent application of these methods for much of the world's population (Meslin et al., 1994).

In North America, Europe, and other developed areas, great reduction in human mortality has been achieved by the virtual elimination of dog-vectored rabies outbreaks by vaccination programs (often compulsory) in association with leash and stray dog measures. Further reduction has accrued from the development and availability in developed areas of modern biologics for preexposure treatment and PET. Other components of the rabies control effort include education of the public and health care professionals regarding exposure avoidance, the proper management of potential exposure to rabies (including animal confinement and observation), prompt and accurate rabies diagnosis, and accessibility to prompt and proper PET or preexposure vaccination when warranted.

Wildlife rabies control by vector population reduction has only rarely proven to be effective, and the North American experience with such measures has not been encouraging (MacInnes, 1988). However, reduction or elimination of wildlife rabies epizootics has proven to be achievable in some situations by wildlife vaccination. Oral rabies vaccination has controlled fox rabies in large areas of Europe and Ontario, Canada. A modified live-virus vaccine has been used in Canada, and efforts in Europe have used baits containing either modified live virus or a recombinant, vaccinia-vectored vaccine. The baits have been distributed by hand, helicopter, or fixed-wing aircraft in one or two distribution campaigns per year in programs that have been continued for many years. The modified live-virus vaccines used in Canadian and European baiting programs do not vaccinate the most common terrestrial rabies vectors of the United States (raccoons and skunks) well by the oral route (Rupprecht et al., 1989). A recombinant vaccinia-rabies glycoprotein vaccine has proven safe and efficacious in laboratory and field trials (Rupprecht et al., 1993) and was licensed by the U.S. Department of Agriculture in 1997 for use in oral rabies vaccination programs conducted by state and federal agencies to control rabies in raccoons. It also has been used with success to control coyote-vectored rabies in south Texas (Fearneyhough, 1998). With millions of vaccine-laden baits distributed annually in North America, and with only a single human infection with the vaccinia recombinant vaccine reported (Rupprecht et al., 2001), the safety of this approach is well established (Slate et al., 2005). Improvements in baits and vaccines could improve the efficiency and efficacy of wildlife vaccination efforts, at the same time making it more cost-effective. Canine adenovirus-vectored (Van Regenmortel et al., 2000) and human adenovirus-vectored (Yarosh et al., 1996) recombinant rabies glycoprotein vaccines are in development and evaluation, and other novel vaccine approaches are proposed (Dietzschold et al., 2003). Advances in wildlife vaccination are now being extended to community dogs in less-developed nations (Rupprecht et al., 2002).

Managing Domestic Animal Exposures to Rabies

Preexposure rabies vaccination of domestic animals is presently achievable with a variety of highly immunogenic and efficacious cell culture vaccines. Those licensed for use in the United States are now all killed-virus vaccines and are listed annually, along with the terms of administration, in the Compendium of Animal Rabies Control that is prepared by the National Association of State Public Health Veterinarians (Sun et al., 2007). Rabies vaccines are presently licensed for use by parenteral inoculation in dogs, cats, cattle, horses, sheep, and ferrets and for use by oral administration in state and federal rabies control programs for raccoons and coyotes. An animal is considered currently vaccinated 1 month after administration of the primary vaccine dose, which establishes a current vaccination status for the remainder of 1 year. A booster dose is required at 1 year after primary vaccination to continue vaccinated status, and that booster and subsequent doses may provide "currently vaccinated" status for up to 4 years, depending on the vaccine and species. Animals that are exposed to rabies that are not current on rabies vaccination status should immediately be euthanized. If the owner is unwilling to have this done, the animal must be strictly isolated for 6 months. Animals that are currently vaccinated should be revaccinated immediately following exposure to rabies.

Human Rabies Prevention

The prompt management of potential human exposure to rabies is a critical component of human rabies prevention. All potential rabies exposures should be evaluated for rabies risk based upon the nature of the contact, the species of animals involved, the circumstances of the incident (e.g., behavior of offending animal, provoked or unprovoked encounter), the current local status of animal rabies, and the adequacy of surveillance. The rabies vaccination status of the animal should not be used to rule out the need for further consideration of rabies transmission (Sun et al., 2007). Not all biting animals need to be killed and tested. A dog, cat, or ferret that has bitten a person but is wanted by the owner and is not demonstrating signs of rabies infection can be confined and observed daily for 10 days. Because of knowledge regarding virus shedding patterns in the days preceding onset of rabies-specific signs (Niezgoda et al., 1997), survival of the animal without rabies onset for 10 days after the bite rules out the need for PEP. If, however, the animal dies or signs of rabies develop during the observation period, it must be immediately euthanized and examined. Similarly, when a 10-day confinement of the offending animal is not possible (if it is symptomatic or has died) or is inappropriate (the offending animal is a wild or exotic species or hybrid), the animal must be humanely euthanized and tested.

The Advisory Committee on Immunization Practices (ACIP) of the U.S. Department of Health and Human Services says that exposure to rabies occurs when infectious virus is introduced into bite wounds or open cuts in the skin or onto mucous membranes. Any penetration of the skin by the teeth, regardless of location, must be considered a bite exposure. Nonbite exposures have occasionally led to rabies infection (Afshar, 1979), with exposure to infectious aerosols or solid organ or corneal transplants from rabies victims carrying the greatest risk. Direct contamination of an open wound (abrasion or scratch) or mucous membrane with saliva or other potentially infectious material (e.g., neural tissue) from a rabid animal is also considered rabies exposure. Contact with blood, urine, or feces (including bat guano) or merely petting a rabid animal is generally not an indication of exposure. Rabies virus in saliva on environmental surfaces is quite labile; therefore, if the material on a surface is dry, it generally can be considered noninfectious (CDC, 2008). Generally, bites or other contact with a rabid animal occurring more than 10 days prior to the recognized onset of signs of rabies in the animal are not considered potential rabies exposures.

Despite the existence of the numerous and widespread terrestrial rabies outbreaks in the country during the period, a surprising 90% of human rabies cases in the United States from 1958 to 2004 have been attributed to bats or bat rabies variants (Dimitrov et al., 2007). From 1953 to 2002, surveillance in the United States had documented at least 39 human cases associated with bat rabies based on the patient's history or virus characterization. In just nine of these, there had been a definite bat bite reported. In another 11, there was known or likely contact with bats associated with an indoor bat encounter under circumstances where a bat bite, with its limited injury in comparison to bites of terrestrial carnivores, may have gone unrecognized (Rupprecht et al., 2002). As a result, the ACIP has developed specific language regarding bat encounters and rabies treatment. "Exposures to bats deserve special assessment because bats can pose a greater risk for infecting humans under certain circumstances that might be considered inconsequential from a human perspective (i.e., a minor bite or lesion)" (CDC, 2008). Furthermore, bat bites may result in very limited injury (Rupprecht et al., 2002), but there is evidence that some bat rabies virus variants may be more likely to be transmitted by superficial, dermal exposures (Feder et al., 1997). One must be cautious not to assume that merely being in close proximity to, or in the same room with, a bat constitutes an exposure. Particular concern must be directed to those situations in which contact was possible but that there is a reasonable probability a bite may have gone unnoticed (Debbie and Trimarchi, 1997). Examples of scenarios justifying consideration of rabies exposure include a sleeping person awakening to find a bat in the room or an adult witnessing a bat in the room with a previously unattended young child or mentally disabled person.

Because it is very often difficult to accurately reconstruct details immediately following an indoor bat encounter and evaluate the likelihood of a "reasonable probability" of exposure, it is prudent to recommend the capture and retention for testing of bats involved in incidents with the potential for human exposure. As rabies positivity rates among insectivorous bats encountered by the general public are typically 3 to 6% (Childs et al., 1994; Mondul et al., 2003), more than 90% of the bats encountered in potential exposure circumstance will test negative for the virus, eliminating the need for further difficult investigations or decisions and avoidable PET. This practice will also help ensure that PET is provided to those who have actually had contact with the relatively small proportion of encountered bats that are rabid (Debbie and Trimarchi, 1997).

Human Rabies Prophylaxis

The relatively long incubation period in rabies infection permits efficacious PET. Modern biologics have afforded the potential for 100% success with proper wound treatment and prompt and appropriately administered immune globulin and a course of vaccine. Postexposure vaccines

have been hypothesized to confer protection largely because they prime an immune response in organs peripheral to the CNS, and the activated lymphocytes, CD4, antibody-secreting plasmocytes, and perhaps antibodies can migrate into the nervous system (Lafon, 2007). An understanding of the nature and sequence of events that confer protection require further investigation. An immediate and thorough washing of bite wounds with soap and water and a viricidal agent are valuable measures for the prevention of rabies (Griego et al., 1995). With the exception of patients with previous immunization, rabies PET should always include passive immunization with human RIG (HRIG) to neutralize virus at the site of exposure and active immunization with vaccine to produce neutralizing antibody that develops in 7 to 10 days after vaccination is initiated. Active immunization also triggers a cell-mediated response that is critical to the success of PET. The importance of the administration of HRIG to the success of PEP has been documented by numerous investigations of PEP failures (Fangtao et al., 1988; Arya, 1999; Sriaroon et al., 2003; Parviz et al., 2004). The HRIG is administered only once, at the beginning of the prophylaxis, but if there is a delay in administering the HRIG, it can be given through the seventh day after the administration of the first dose of vaccine. The recommended HRIG dose is 20 IU per kilogram of body weight. If anatomically feasible, the full dose should be infiltrated into and around the wounds. The remaining volume or, for treatments for exposures with no recognizable wounds, the entire dose, should be injected intramuscularly at one or more sites distant from the site of vaccine inoculation. Two immunologically comparable vaccines grown in two differently derived cell culture systems are currently licensed for use in the United States: a human diploid cell vaccine (HDCV) and a purified chick embryo cell vaccine. Both are packaged for and administered as a 1-ml intramuscular dose for pre- or postexposure administration. For PET, either of the vaccines can be used in a 5-dose course, with 1-ml intramuscular injections given in the deltoid area (or for small children, the anterolateral aspect of the thigh) on days 0, 3, 7, 14, and 28, commencing as soon as possible after the exposure is known. PET for previously vaccinated individuals is comprised solely of 2 doses of vaccine administered as a 1.0-ml intramuscular injection in the deltoid region on days 0 and 3, commencing as soon as possible after the exposure is known. Two HRIG products are licensed for use as well, and both are anti-RIG (IgG) preparations concentrated by cold ethanol fractionation from plasma of hyperimmunized human donors. HRIG is not administered to previously vaccinated patients. For this purpose, previously vaccinated status applies only to individuals who have received one of the recommended pre- or postexposure regimens of the currently licensed vaccines or who received another vaccine or regimen and had a documented adequate neutralizing antibody titer (0.5 IU or greater or complete neutralization at 1:5 serum dilution on the RFFIT).

Although several other efficacious cell culture vaccines are widely available outside the United States, nervous tissue vaccines and immune serum of equine origin are still employed in some areas. A purified RIG of equine origin has been used effectively in developing countries (Wilde et al., 1989). Several alternative PET schedules are employed outside the United States, including multisite regimens that are accelerated by administration of more than one dose on the first day of treatment, by either intramuscular or intradermal inoculation. These regimens may induce early antibody response, which is beneficial where RIG is not available, and can reduce the amount of scarce vaccine required (Dreesen, 1997).

Preexposure rabies vaccination is available for persons at risk of rabies exposure, such as veterinarians, animal control officers, animal handlers, rabies laboratory workers, others whose activities bring them into contact with a rabies vector species, and certain travelers. Preexposure vaccination does not preclude the need for PET following a known exposure but eliminates the need for RIG and reduces the number of vaccine doses to two. Furthermore, a previously vaccinated individual might be protected from unrecognized exposures or when PET is unavoidably delayed (CDC, 2008). The primary preexposure regimen consists of three 1-ml intramuscular injections, given one-each on days 0, 7, and 21 or 28. Depending on the risk category, booster vaccinations may be recommended when rabies neutralizing antibody titer is less than 0.5 IU or less than complete neutralization at a 1:5 serum dilution by RFFIT.

Routine determination of adequate serum rabies neutralizing antibody titer following primary pre- or postexposure vaccination is not required unless the person is immunosuppressed. Persons at the highest or continuous risk of inapparent rabies exposure, such as those working in rabies research or vaccine production laboratories, should have a titer determination every 6 months (CDC, 2007). Others in the frequent-risk category, such as those working with mammals in areas where rabies in animals is enzootic, should be serologically tested at 2-year intervals (Briggs and Schwenke, 1992). A single booster vaccination is recommended when the titer is less than complete neutralization at 1:5 serum dilution on the RFFIT or 0.5 IU. The ACIP also recommends that certain travelers and animal workers in areas with low animal rabies rates are in an infrequent exposure category and do not require routine preexposure booster doses following primary vaccination (CDC, 2008).

Reactions following vaccination with the currently licensed cell culture vaccines and administration of HRIG are less serious and less common than with previously available biologics. Mild local reactions such as pain, erythema, and itching are not uncommon and are reported in 30 to 74% of vaccinees (Noah et al., 1996; Briggs et al., 2000). Mild systemic reactions including headache, nausea, abdominal pain, muscle aches, and dizziness are reported in 5 to 40% of recipients. Guillain-Barre syndrome-like neurologic illness and other central and peripheral nervous system disorders temporally associated with HDCV administration have not been linked in a causal relationship with the vaccination (Tornatore and Richert, 1990). A delayed (2 to 21 days postinoculation) immune complex-like reaction was reported among 6% of patients receiving booster doses of HDCV. The reaction included hives sometimes accompanied by arthralgia, arthritis, angioedema, nausea, vomiting, fever, and malaise, but the reaction has not been life-threatening (Dreesen et al., 1986). No fetal abnormalities have been associated with rabies vaccination, and pregnancy is not a contraindication for rabies vaccination (Chutivongse et al., 1995).

RESEARCH DIRECTIONS

At its simplest, there are two remaining ultimate goals of rabies research: the development of a cure for the disease after symptoms develop and the eradication of the virus. Although eradication would make the need for a cure moot, worldwide elimination of the virus may be relatively unattainable because of the myriad of variants of rabies and

rabies-related viruses that cycle in numerous and changing terrestrial and chiropteran vectors.

We now have effective means to combat rabies transmission to humans. Rapid and reliable postmortem diagnosis of rabies in biting animals is readily achievable. Highly effective and safe biologics exist for the prevention of rabies in humans and domestic animals by pre- and postexposure vaccination. Efficacious dog vaccines and stray dog control programs have been demonstrated to effectively reduce human rabies deaths to rare events by eliminating the domesticated dog as a significant vector of the disease, as has occurred across North America, Europe, and most recently, large areas of Latin America. Encouraging evidence is mounting for the control of rabies outbreaks in wildlife over large areas by oral rabies vaccination strategies employing vaccine-laden baits distributed in the environment. Sadly, resource limitations, and to a lesser degree lack of a political will and societal resistance, have prevented successful implementation of these strategies in the areas of the world accommodating the vast majority of the world's population. As a result, human mortality from rabies infection is still commonplace in all but a relatively small number of developed nations. Nerve tissue vaccines, which are associated with much higher rates of postvaccinal adverse events that far outweigh their one advantage of low cost, still constitute 25% of all human rabies vaccine produced worldwide (Dreesen and Hanlon, 1998) and produce significant mortality and disability (Knobel et al., 2005). The efforts to muster the resources required to effect affordable rabies control programs worldwide can benefit from economic analysis to make optimal resource allocation decisions (Meltzer and Rupprecht, 1998).

Sufficient resources will remain unattainable for a successful cosmopolitan rabies control program if the present costs to produce, store, and deliver therapeutic biologics are not drastically reduced. Consequently, a priority research imperative for rabies control is the development of safe, effective, inexpensive, and mass-producible products for active and passive immunization. Recombinant vaccine has already been instrumental in rabies control, as a vaccinia-rabies glycoprotein recombinant has been used in oral rabies vaccination programs in Europe and the United States (Brochier et al., 1996). Newly developed genetically engineered oral rabies vaccines must be evaluated in the regionally important rabies vector species (Blanton et al., 2006). The vaccination of free-ranging wildlife and dogs could be greatly simplified with nonpathogenic vector viruses that are transmitted horizontally by host-to-host spread of infection or vertically in the host population by transgenic technology. The transgenic approach has already been utilized to produce tomatoes expressing rabies virus G protein (McGarvey et al., 1995). Studies have demonstrated that mice immunized orally with plant-derived rabies virus-specific antigen can be protected from rabies challenge (Yusibov et al., 2002).

Recombinant vaccine technology offers hope of multivalent childhood vaccines protecting against numerous infectious diseases, and it has been suggested that this could be incorporated into childhood vaccination programs in areas of high rabies endemicity where postexposure vaccination is not readily accessible (Dreesen, 1997). Nonreplicating recombinant avian poxvirus constructs expressing rabies virus glycoprotein have been demonstrated to effectively produce rabies immunity (Taylor et al., 1995; Fries et al., 1996) and have been observed to not produce adverse signs of infection or disease in animals following inoculation (Taylor et al., 1991). Multivalent recombinant vaccines for the immunization of animals have been developed (Hu et al., 1997) and licensed for use in cats (Sun et al., 2007). Human adenovirus-rabies glycoprotein recombinant vaccines have been shown to achieve good responses by subcutaneous, intramuscular, and even oral routes in mouse models (Xiang et al., 2003; Zhou et al., 2006). Another possible way to reduce the cost of production and increase stability of the immunizing product as well as possibly confer lifelong duration of immunity from infant vaccination is the development and use of DNA vaccines (Butts et al., 1998). This technology utilizes the observation that introduction of a plasmid carrying a reporter gene into an animal results in the expression of that gene in vivo, the DNA persists for long periods, and the protein produced by the plasmid can induce an immune response (Babiuk et al., 1999). Rabies DNA vaccines have been developed and preliminary efficacy trials in nonhuman primates have demonstrated a capability to induce protective antibody (Lodmell et al., 1998; Lodmell et al., 2002).

Although evidence is convincing that infiltration of the exposure site with neutralizing antibody is necessary for optimal protection by PET, availability prevents the consistent use of passive protection with PET throughout much of the world. HRIG is used exclusively in the United States, but a purified immune globulin of equine origin is available that is used widely elsewhere and has been well tolerated. The use of monoclonal antibody technology may permit the production of an affordable and consistent alternative to avoid the costs and acute shortages associated with antiserum-derived immunoglobulins. Monoclonal antibodies of human and mouse origin are being shown to possess significant protective activity in evaluations in vitro (Bakker et al., 2005) and in PEP animal models (Hanlon et al., 1998; Sloan et al., 2007).

The use of antibody therapies, antiviral compounds, or interferon and its precursors have shown some promise as a cure for rabies in vitro, but with a single prominent exception, have proven futile when utilized in vivo during clinical rabies. The survival of rabies infection of a teenage girl in the United States following a therapy of induced coma and use of multiple antiviral compounds in 2004 is an exciting event, but it is still very unclear what promise it portends for repeated success. Novel methods to identify antiviral targets for discovery of therapeutic drugs have been proposed (Wunner et al., 2004). Expectations for reversal of the disease process once symptoms begin will be very difficult without a greater knowledge of pathogenesis and pathologic mechanisms of the infection at the host and cellular levels. One interesting proposed strategy is the use of antisense oligodeoxynucleotides, complimentary to rabies virus genomic RNA. If rabies pathogenesis is due to virus replication in neural cells leading to suppression of host gene expression, it has been hypothesized that exposure to antisense sequences might reverse the disease process (Fu, 1997). Preliminary evidence in vitro suggests that one such oligdeoxynucleotide can almost completely block rabies virus infection of cells and inhibit cell-to-cell spread of rabies virus in mouse neuroblastoma cell culture (Fu et al., 1996).

In the United States, 32 of 35 indigenously acquired human rabies cases from 1958 to 2000 resulted from infection with bat rabies viruses associated with bats. Bat rabies viruses were implicated in 26 of the 28 of those in which there was no clear history of a bite (Messenger et al., 2002). In 12 of those cases, there was some physical contact with a

bat without an evident bite. In four others, there had been one or more indoor encounters with a bat with no contact recognized. Bat teeth are small and sharp, and a bat bite may not draw blood or be noticed (Rupprecht, 1996). These observations have prompted the changes in the guidelines for the management of potential rabies exposures following encounters with a bat. What may be even more puzzling than the cryptic nature of the recent human rabies cases is the identification in the vast majority of these cases of rabies viruses associated only with two species of bats that are very rarely encountered by humans. In 19 of the 26 bat rabies human deaths in the United States during the period, the variant has been identified by molecular typing as that associated with silver-haired and pipistrelle bats (Messenger et al., 2002). These species are noncommensal bats that are very rarely seen and even more rarely involved in recognized contact with the public (Childs et al., 1994). Preliminary investigations have suggested that the silver-haired–pipistrelle bat rabies variant may possess special growth characteristics related to temperature and nonneural cell adaptation with possible implications for transmission by superficial intradermal exposures (Moromoto et al., 1996). These data invite further investigation of the cryptic nature of the transmission of rabies to humans in the United States and of the preponderance of bat rabies, particularly this variant.

The tremendous reduction in the risk of PEP-associated adverse reactions realized with modern cell culture vaccines and immune globulin of human origin resulted in a dramatic increase in the number of PEPs in the United States in the early 1980s. The increase of rabies in densely populated areas of the mid-Atlantic and northeastern states due to the raccoon rabies epizootic and expansion of treatment criteria to include certain nonbite exposures have accounted for enormous increases there in the numbers of rabies treatments (Wyatt et al., 1999). The recommendation that "any potential exposure to a bat requires a thorough evaluation" for the need for prophylaxis (CDC, 2008) may have further escalated shortages of biologics and the annual costs for human vaccination in this country. Applied research on the epidemiology of human rabies PEP is required to verify the appropriateness of current treatment algorithms. This is particularly true in light of realistic risk assessments and the consideration of the opportunity costs of the expenditures that would be required to try to bring the already low rabies mortality rate in the United States to zero (Rupprecht et al., 1996).

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Arboviruses

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23

LABORATORY PROCEDURES FOR DETECTING VIRUSES

Introduction

The term arbovirus is a contraction of "*ar*thropod-*bo*rne *virus*" and has no phylogenetic or classification significance. This term describes the mechanism by which these viruses are transmitted and maintained in nature: through the bite of a hematophagous arthropod. Most medically important arboviruses are transmitted by either mosquitoes or ticks. In the United States alone, representatives from at least five virus families can be transmitted by biting arthropods (Table 1). This review will focus on the medically important arboviruses.

Because these viruses are transmitted by arthropods, arboviral disease usually manifests itself during the warmer months in the temperate climates of the world. Arboviral disease can, however, be contracted in the winter months in milder climates, and disease transmission can occur year round in the tropics. During the milder times of the year, or depending on the patient's travel history, testing for arboviruses should be included in the laboratory diagnosis of cases compatible with arboviral infections.

There are 535 arboviruses listed in the International Catalogue of Arboviruses (Karabatsos, 1985), but most have not been associated with human disease. Continued encroachment on the world's tropical rainforests, however, coupled with rapid transport of humans and animals, makes arboviruses emerging and reemerging pathogens. This observation means that new arboviruses may be associated with human diseases or known arboviruses may cause outbreaks in previous or new locales. The discovery of West Nile (WN) virus (WNV) in the United States in 1999 is a recent example of arbovirus movement. Identification of emerging agents will, by definition, be difficult, with the medical and veterinary community depending on specialty reference laboratories capable of working with and identifying these biosafety level 3 and 4 pathogens (Centers for Disease Control and Prevention, 1993). The World Health Organization (WHO) sponsors a laboratory network of WHO Collaborating Centers distributed throughout the world, which specialize in diagnosing arboviral diseases. It is likely that a clinical sample from an arbovirus infection will end up at one of these laboratories for diagnosis or confirmation.

History

Yellow fever (YF) epidemics probably occurred as early as 1648 in the Yucatan Peninsula of Mexico. *Aedes aegypti* mosquitoes, which are the urban vectors of YF, also transmit dengue (DEN) virus, the cause of DEN fever. DEN fever outbreaks occurred quite frequently in the southern United States until the 1920s, when populations of the vector mosquito were controlled. Both DEN and YF continue to occur in tropical America and Africa, even though an effective YF vaccine exists. This inability to control YF despite the availability of an effective vaccine reflects the poor economic conditions of the countries where YF is endemic, where this vaccine is still too expensive for general use (Monath, 1991). DEN virus also causes DEN hemorrhagic fever (DHF) and DEN shock syndrome (DSS), which currently occur as major, lethal epidemics of children in Southeast Asia and appeared for the first time in the New World in Cuba in 1981 (Kouri et al., 1983; Guzman et al., 1984; Guzman et al., 1990).

The primary clinical manifestation of life-threatening arboviral disease in North America has been encephalitis. Three mosquito-borne viruses that cause human encephalitis were discovered during the 1930s. Western equine encephalitis (WEE) virus was isolated in 1930 from horses (Meyer et al., 1931) and in 1938 was associated with encephalitis in humans in California. It now occurs infrequently in the irrigated farmland of the western United States and Canada. Eastern equine encephalitis (EEE) was isolated in 1933 from horses (TenBroeck and Merrill, 1933). It was subsequently isolated from people in 1938. Currently, EEE has a distribution throughout most of the eastern half of the United States. The first outbreak of St. Louis encephalitis (SLE) virus occurred in 1933 in St. Louis, MO, with 1,095 reported cases (Cumming, 1935). The last major SLE epidemic was in 1975, with 1,815 reported cases. Endemic (rural) SLE may occur each year in much of the western United States (Monath and Tsai, 1987; Tsai et al., 1987b; Reisen et al., 1990; Reisen et al., 1992a; Reisen et al., 1992b; Reisen and Chiles, 1997). It has been hypothesized that major urban SLE outbreaks occur every 7 to 10 years; however, this no longer appears to be the case. The reduction in the incidence of SLE may be due to human lifestyle modifications, such as the use of air conditioning and television. Focal outbreaks can occur each year; however, many times they are localized to the poorer

TABLE 1 Medically important arboviruses in the United States

Virus family	Pathogen	Related virus(es)
Togaviridae	EEE virus	HJ virus
	WEE virus	
	VEE virus	
Flaviviridae	WNV	SLE virus
	SLE virus	WNV
	POW virus	
	DEN virus	
Bunyaviridae	CAL serogroup viruses	Many
	LAC encephalitis virus	
	CAL encephalitis (CE)	
	SSH virus	
	JC virus	
	CV virus	
Reoviridae	CTF virus	None
Rhabdoviridae	VSV	Rabies virus

urban areas in scattered locations such as Chicago, Philadelphia, Houston, and New Orleans.

Two other encephalitis viruses, WNV and Venezuelan equine encephalitis (VEE) virus, have been associated with major epidemics or are maintained in nature in enzootic cycles. The varieties of VEE viruses associated with these differing epidemiologic presentations can be separated both serologically and through genetic analysis. VEE virus has caused major human epidemics periodically throughout Central and South America since the 1930s, the most recent in 1995 in Colombia and Venezuela (Kinney et al., 1989; Sneider et al., 1993; Weaver et al., 1996; Rivas et al., 1997; Kinney et al., 1998). It is now believed that the earliest VEE epidemics were caused by incompletely inactivated vaccines (Sneider et al., 1993; Weaver et al., 1999). Current VEE epidemics are caused by epidemic strains of VEE virus thought to have evolved from naturally occurring enzootic VEE viruses (Rico-Hesse et al., 1995; Powers et al., 1997; Kinney et al., 1998). The reasoning for this is derived partly from the inability to isolate epidemic VEE viruses during interepidemic periods.

Following the discovery of WNV in the New York City area in 1999, it has now become the leading cause of vector-borne human encephalitis in the United States. WNV has spread throughout the continental United States and Canada, and there is serological evidence for WNV activity in Mexico, Central and South America, and the Caribbean. It is of interest that a variety of novel modes of transmission of WNV have been either suggested or proven (Iwamoto et al., 2003; Avalos-Bock, 2005; Busch et al., 2005; Hoekstra, 2005; Kusne and Smilack, 2005; Kuehn, 2006; Lee and Biggerstaff, 2006; Montgomery et al., 2006; O'Leary et al., 2006; Hinckley et al., 2007). These modes include blood, transplanted tissue, and human breast milk (transmission to infants through the milk of infected mothers).

Detailed reviews for all of these viruses as well as a currently emerging encephalitis caused by the California (CAL) serogroup virus, La Crosse (LAC) encephalitis, are recommended for further study (Calisher and Thompson, 1983; Monath, 1988, 1996; Trent et al., 1989; Tsai and Monath, 1996: McJunkin et al., 1998).

Methods Used

General Considerations

Laboratory diagnosis of arboviral infections has traditionally been based upon serological identification of antiviral antibodies and/or isolation of virus. While the classical serological assays of hemagglutination inhibition (HI), complement fixation (CF), and neutralization (NT) of virus infectivity have been replaced by enzyme-linked immunosorbent assay (ELISA), each of these earlier tests still have applicability. The timing after infection of certain viral infections can sometimes be ascertained with the CF test. While the laboratorian can readily distinguish between virus families (e.g., flaviviruses and togaviruses), within an individual family, many of the viruses are so closely related antigenically that only the virus NT test can differentiate infections.

While the expensive technique of virus isolation by inoculation into susceptible cell culture is losing ground to more rapid assays like PCR and antigen-detection ELISA, the former approach is still useful. For example, alphaviruses replicate in common continuous cell cultures like Vero or BHK-21 cells, often demonstrating virus-specific cytopathic effects within 24 hours. These virus-infected cells can then be used to identify the infecting agent by indirect immunofluorescence assay (IFA) using well-characterized virus-specific murine monoclonal antibodies (MAbs). Very few PCR assays developed for arboviruses have been critically and completely analyzed to the extent that they now function as simple and reproducible lab tests. The caveat with all newer assays that detect only viral protein or nucleic acid is their inability to produce replicating virus useable for future serologic or genetic analysis.

Basic Principles

A historical analysis of arboviral infections investigated at the WHO Collaborating Centers for arboviruses in the Division of Vector-Borne Infectious Diseases, U.S. Centers for Disease Control and Prevention (CDC), identified the viruses that cause enough disease to warrant their inclusion in routine diagnostic virology testing panels. These panels include representatives from all virus families and can be organized by their geographic distribution. The decision of which virus panel is used can be based upon the patient's location and travel history (Fig. 1). These viruses are not the only arboviral agents responsible for disease; but rather, these virus panels should detect the majority of arboviral infections. Regardless of the assay employed, confirmation of arboviral infection requires acute- and convalescent-phase serum samples that yield a demonstrable increase in antiviral antibody activity. Introduction of ELISA protocols that measure virus-specific immunoglobulin M (IgM), especially when applied to acute-phase serum or cerebrospinal fluid (CSF) samples, yield good approximations of recent infections when the timing after infection of the specimen is appropriate. For some arboviruses, however, IgM reactivity can be measured weeks after the onset of disease. For most arboviruses, serologic cross-reactivity with related viruses increases as the infection progresses. Because of this and the close antigenic relatedness of many of the agents within the same virus family, IgG ELISAs are often not very specific. Regardless of this, it is a simple matter to differentiate viruses from different families (e.g., togaviruses from flaviviruses). Because epitopes that elicit virus-neutralizing antibody are under the most severe immunologic pressure, these epitopes are usually the most virus specific. Consequently, the virus NT assay demonstrates a fair amount of serologic specificity, even with convalescent-phase serum samples.

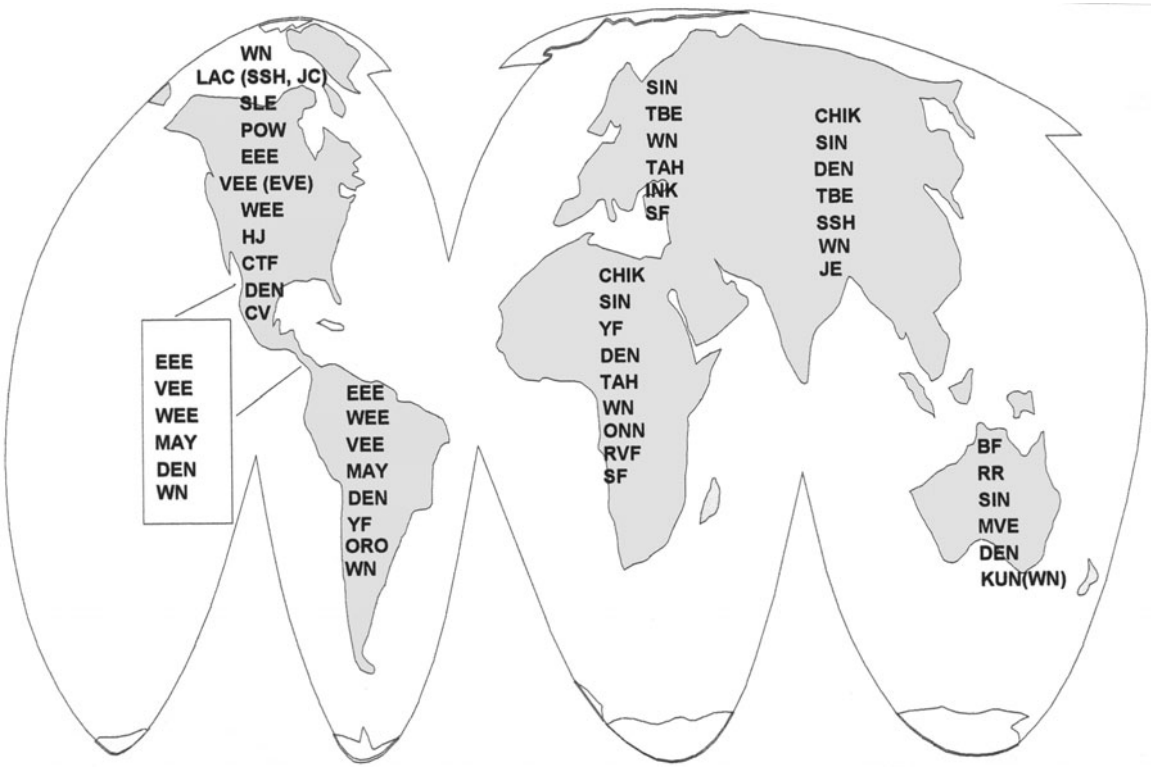

FIGURE 1 Antigen panels for arboviral testing based upon geographic distribution and prevalence. Abbreviations: EVE, Everglades VEE; SF, Semliki Forest; SIN, Sindbis; TAH, Tahyna; INK, Inkoo; RVF, Rift Valley fever; ORO, Oropouche; KUN, Kunjin.

Applications

Serology for Antibody Testing

HI

The HI test measures the ability of antiviral antibody to block the virus capacity to agglutinate erythrocytes (Clarke and Casals, 1958). This was the first technique used to characterize arboviruses. The HI test successfully differentiated togaviruses (group A arboviruses, primarily alphaviruses) from flaviviruses (group B arboviruses) long before modern biochemical techniques confirmed this observation. Many laboratories still utilize the HI test, although with the advent of ELISA, it is being replaced. The HI test requires preparation of tedious hemagglutination buffers, continual test standardization, and the routine availability of gander erythrocytes. As the disease progresses, virus cross-reactivity in the HI test also increases. It is not uncommon for convalescent-phase serum samples to react with two or more virus antigens within the same virus family, making even a fourfold or higher serum HI titer rise between acute- and convalescent-phase serum samples difficult to interpret.

CF

The CF test measures the ability of the antiviral antibody to fix complement in the presence of virus antigen. Quite possibly, it is more difficult to maintain proper quality control of the CF test than the HI test. Consequently, it is used in only special situations, such as attempting to determine the timing after infection of an individual serum sample (Monath et al., 1980). Because CF antibody appears later in infection but has a shorter half-life (around 2 years), this test has been used as an indicator of more recent primary infection. With the advent of the IgM ELISA, allowing for direct measurement of the early IgM antibody, CF tests are not as useful. The CF test may, however, indicate a recent infection if the serum sample is taken after the IgM antibody has waned.

PRNT

The plaque-reduction neutralization test (PRNT) is a contradiction among assays used to diagnose arboviral infection. It is by far the most expensive and problematic test to perform, but it is still the only serologic assay able to reliably differentiate infection between two closely antigenically related viruses. The subtlety of the PRNT is based upon the plaquing requirements of various arboviruses. Plaquing of arboviruses is usually performed in a variety of continuous mammalian cell lines. The most common of these are Vero, BHK-21, and CER cells. Both plaque size and morphology might differ, depending on the cell type used. Time to plaque formation also varies. Flaviviruses may take 7 to 10 days for plaques to form, while alphaviruses usually plaque in 24 to 48 h. To perform the PRNT, a virus seed of known titer must be available. Since many arboviruses lose titer upon freeze-thawing, it is best to have multiple aliquots of the virus seed. A constant amount of virus (50 to 100 PFU) is mixed individually with dilutions of the serum being tested. Following plating on cells, plaques are visualized by adding a solution of the vital dye neutral red. The number of plaques in an individual plate is then divided by the starting number of virions to calculate a percent neutralization. Typically, the PRNT is interpreted at a 70% PRNT titer, that is, the last dilution of serum that inhibits 70% of the total added plaques.

MAC-ELISA

Currently, the ELISA is used to measure either IgM or IgG individually. As with other infections, IgM titers usually signify recent virus infection. While many IgM protocols have been designed over the years, the most appropriate protocol for measuring IgM is the IgM-capture ELISA (MAC-ELISA) (Westaway et al., 1974; Heinz et al., 1981; Burke and Nisalak, 1982; Jamnback et al., 1982; Monath et al., 1984; Bundo and Igarashi, 1985; Burke et al., 1985a; Burke et al., 1985b; Calisher et al., 1985a; Calisher et al., 1985c; Carter et al., 1985; Dykers et al., 1985; Calisher et al., 1986a; Calisher et al., 1986b; Calisher et al., 1986c; Besselaar et al., 1989; Cardosa et al., 1992; Sahu et al., 1994; Kittigul et al., 1998; Martin et al., 2002; Martin et al., 2004). This approach minimizes the interference of the higher-avidity IgG with IgM binding to antigen and consequently is more sensitive than the indirect ELISA format for IgM (Heinz et al., 1981). The capture design also permits use of antigen from a variety of sources, including those that normally have too much irrelevant protein for direct coating of plates. In the MAC-ELISA, human antiviral antibody is first captured into a 96-well ELISA plate by precoated commercial anti-human IgM antibody. The virus specificity of this captured IgM is determined by reacting individual wells with different virus antigens. The captured virus antigen is then detected with an antiviral antibody.

The most efficient MAC-ELISA design uses broadly cross-reactive murine MAbs, conjugated to enzyme as antiviral antigen detector molecules. Three of these MAbs—2A2C-3 (broad alphavirus reactor), 6B6C-1 (broad flavivirus reactor), and 10G-4 (broad bunyavirus reactor)—are currently used to identify viral antigens from these three virus families (Table 2) (Roehrig et al., 1983; Roehrig et al., 1990a; Ludwig et al., 1991). Since the absorbance recorded in this ELISA is dependent upon the amount of antiviral antibody in the sample (provided that antigen is in excess), this ELISA can be run first at a single screening dilution (e.g., 1:400). The results from the MAC-ELISA are usually interpreted by dividing the absorbance of the test sample on antigen (*P*) by the absorbance of a negative control serum on antigen (*N*). In our laboratory, *P*/*N* ratios of ≥2.0 are considered positive, with the caveat that *P*/*N* values between 2 and 3 are often false positives. In this case, another serological assay (e.g., PRNT) should be performed to confirm equivocal results. Alternatively, a convalescent-phase serum can be tested. The antibody titer in this specimen should have increased from that of the acute-phase specimen. The MAC-ELISA is capable of distinguishing among infections caused by the medically important alphaviruses (EEE, WEE, and VEE). Commercial IgM enzyme immunoassay kits are now being produced. There are currently U.S. Food and Drug Administration-approved and commercially available MAC-ELISAs to detect WNV infection. The microplate-based MAC-ELISA has been adapted to lateral-flow tests in dipstick or cassette format. This makes the test qualitative rather than quantitative but permits rapid field testing of specimens (Sathish et al., 2002; Prince et al., 2005; Niedrig et al., 2007; Rawlins et al., 2007; Sambol et al., 2007).

IgG ELISA

Standard indirect IgG ELISA can be used with arboviruses (Frazier and Shope, 1979; Roehrig, 1982). The problem with this approach is the wide variety of agents causing these diseases. It is simply too difficult and time-consuming to prepare pure virus antigen for even the limited subset of arboviruses used in antigen panels. To circumvent this problem, an indirect IgG ELISA has been developed in which virus antigen is captured into wells with broadly cross-reactive murine MAbs for each of the virus families (Table 2) (Johnson et al., 2000). These MAbs are first coated to wells, and then the appropriate viral antigen is added. After virus antigen has been captured, this IgG functions like any other indirect ELISA. Immune serum samples from infected individuals (infection immune) are added, and the binding of antiviral antibody is detected with a commercial anti-species antibody conjugated to enzyme. While antigen is typically prepared as virus-infected mouse brain that has been processed to remove nonspecific inhibitors, virus-infected cell culture fluids also can be used. The latter antigen is typically lower in activity. There are commercial enzyme immunoassay kits available; however, their reliability has yet to be conclusively proven.

Blocking ELISA

With the discovery of WNV in the United States and the subsequent identification of a wide range of virus-infected animals, a new test was designed that did not rely on species-specific antibodies for performance. This blocking ELISA is based upon the ability of infection immune sera to block the binding of reporter virus-specific MAbs to virus antigen (Blitvich et al., 2003a; Blitvich et al., 2003b; Jozan et al., 2003). A similar test had been developed previously for the Australian flavivirus, Murray Valley encephalitis (MVE) virus (Hawkes et al., 1990). The blocking ELISA has been used successfully with avian and equine sera; however, it has not been as useful for human serum samples for an as yet undefined reason.

Microsphere Immunoassay

The MAC-ELISA and IgG ELISA for some arboviruses have been adapted to microsphere immunoassay (e.g., Luminex). This rapid flowthrough assay design is based upon microparticles containing mixtures of chromophores. The wide range of chromophore mixtures available allows this approach to be multiplexed with more than one antigen. For now, this approach has been applied to the flaviviruses WNV and SLE virus and is based upon the reactivity of antibodies with either the envelope (E) glycoprotein or the NS5 nonstructural protein (Wong et al., 2004; Johnson et al., 2005). Eventually, additional viruses will be added to the antigen cocktail, which should permit testing for a wide variety of viruses using only a single serum specimen.

IFA Test

One of the oldest commercial assays for antibody to SLE, WEE, EEE, and LAC viruses is based upon end point titration of sera by IFA. This kit is used by many public health and commercial labs. Since this is an indirect format, the problems of IgG competition for IgM binding occur in the IgM IFA test. The IFA test lacks both the sensitivity and quantitative characteristics of the ELISA. For this reason, serological diagnosis based upon IFA titrations is not preferable.

Virus Isolation and Identification

Three approaches are currently used to identify virus in complex solutions. The oldest method is to inoculate specimens into susceptible cell cultures, wait for virus-specific cytopathic effects, and then identify the virus isolate by a complex serologic testing scheme. More recent techniques use antiviral antibody to "capture" virus antigen from a solution to be later identified with antiviral antibody. While both of these assays rely on viral proteins for the identification process,

TABLE 2 MAbs useful in arbovirus identification

Virus MAb	Virus specificity	Utility for[a]: ELISA MAC	ELISA IgG	ELISA Ag capture[d]	IFA	IHC[b]	Reference[c]
Alphaviruses							
VEE virus							
1A2B-10	Wild-type VEE	–	–	D	+	+	Roehrig et al., 1991
5B4D-6	TC-83 VEE	–	–	D	+	+	Roehrig et al., 1982
1A3A-5	1AB, 1C, 2	–	–	D	+	+	Roehrig and Mathews, 1985
1A4D-1	1AB, 1C, 1D	–	–	D	+	+	Roehrig and Mathews, 1985
1A1B-9	1D, 1E, 1F	–	–	D	+	+	Rico-Hesse et al., 1988
1A3A-9	All subtype 1	–	–	D	+	+	Roehrig and Mathews, 1985
1A3B-7	All VEE complex	–	–	D	+	+	Roehrig and Mathews, 1985
WEE virus							
2B1C-6	WEE	–	–	D	+	+	Hunt and Roehrig, 1985
2A3D-5	WEE complex	–	–	C	+	+	Hunt and Roehrig, 1985
2D4-1	HJ	–	–	D	+	+	Karabatsos et al., 1988
2A2C-3	All alphaviruses	+	–	D	+	+	Karabatsos et al., 1988
EEE virus							
1B5C-3	NA EEE[e]	–	–	D	+	+	Roehrig et al., 1990a
1B1C-4	EEE complex	–	–	D	+	+	Roehrig et al., 1990a
1A4B-6	All alphaviruses	–	+	C	+	+	Roehrig et al., 1990a
Flaviviruses							
SLE virus							
6B5A-2	SLE	–	–	D	+	+	Roehrig et al., 1983
4A4C-4	SLE	–	–	C	+	+	Roehrig et al., 1983
6B6C-1	All flaviviruses	+	–	D	+	+	Roehrig et al., 1983
JE virus							
JE314H52	JE	–	–	D	+	+	Unpublished
6B4A-10	JE complex	–	–	C	+	+	Guirakhoo et al., 1992
6A4D-1	JE, MVE	–	–	D	+	+	Guirakhoo et al., 1992
MVE virus							
4B6C-2	MVE	–	–	D	+	+	Hawkes et al., 1988
YF virus							
5E3	YF	–	–	D	+	+	Schlesinger et al., 1983
2D12	YF	–	–	D	+	+	Schlesinger et al., 1983
864	Vaccine YF	–	–	D	+	+	Gould et al., 1985
117	Wild-type YF	–	–	D	+	+	Gould et al., 1989
DEN virus							
D2-1F1-3	DEN1	–	–	D	+	+	Unpublished
3H5-1-21	DEN2	–	–	D	+	+	Henchal et al., 1985
D6-8A1-12	DEN3	–	–	D	+	+	Unpublished
1H10-6-7	DEN4	–	–	D	+	+	Henchal et al., 1982
4G2	All flaviviruses	–	+	C	+	+	Henchal et al., 1982
Bunyaviruses							
LAC virus							
807-18	LAC	–	–	D	+	+	Gonzalez-Scarano et al., 1982
10G4	CAL group	+	+	C/D	+	+	Ludwig et al., 1991

[a]+, useful; –, not useful.
[b]IHC, immunohistochemistry.
[c]Reference in which MAb was first described. Publication lists all important biological characteristics of MAb.
[d]Used as capture (C) or detector (D) antibodies. Ag, antigen.
[e]North American (NA) EEE viruses only.

the advent of PCR assays has now established genome identification as one of the primary tests for virus identification. The evolution of rapid genome sequencing and the accumulation of large numbers of virus gene sequences have allowed PCR identification to evolve into a precise virus identification procedure. For those labs that do not have PCR capability, the development of virus-specific MAbs has improved the classic techniques of virus isolation and serologic identification to the extent that these approaches are still viable options for the clinical virology laboratory.

IFA Identification

Previously, virus NT assays were necessary to differentiate closely related viruses such as flaviviruses. There now exist MAb reagents capable of identifying a specific virus by IFA (Table 2) (Roehrig, 1986, 1990; Heinz and Roehrig, 1990; Roehrig and Bolin, 1997). There are also MAbs capable of identifying virus complexes and even larger virus groups (e.g., all alphaviruses or all flaviviruses). While these MAbs have replaced virus-grouping antisera prepared by the National Institutes of Health (NIH), the NIH grouping serum samples are still quite useful in characterizing those arboviruses for which few MAbs are available (e.g., bunyaviruses). Because of the short supply of the NIH grouping sera, these reagents are usually available to reference laboratories, whereas the MAb reagents are available to all public health laboratories and can identify all domestic medically important arboviruses.

Antigen-Capture ELISA

Because of the high avidity and precise specificity of MAbs, these reagents are currently being fashioned into antigen-capture ELISA protocols (Hildreth et al., 1982; Beaty et al., 1983; Hildreth and Beaty, 1983; Hildreth et al., 1984; Kuno et al., 1985; Monath et al., 1986; Scott and Olson, 1986; Tsai et al., 1987a; Tsai et al., 1988; Gajanana et al., 1995; Brown et al., 1996; Hunt et al., 2002). In these assays, viral proteins are immobilized onto a solid phase by an antiviral MAb. This captured antigen is then detected by using an antiviral antibody conjugated to enzyme. For simplicity, the detecting MAbs are usually broadly cross-reactive, such as the flavivirus MAb 6B6C-1. This approach reduces the number of enzyme conjugates necessary for virus identification. These protocols are currently formulated in ELISA format, but some have been redesigned for commercial use as dipstick or lateral-flow assays (Ryan et al., 2003) The dipstick assays have been particularly useful in mosquito surveillance efforts, where smaller number of specimens are routinely tested, and the test is more amenable to field analyses. Currently, antigen-capture ELISA has been developed for EEE, WEE, SLE, LAC, WN, and DEN viruses. While there have been no protocols published for VEE antigen detection, the MAbs are available, and development of this assay should not be far off. MAbs useful in antigen-capture ELISA, as either capture or detector antibodies, are included in Table 2.

NAAT

A variety of nucleic acid amplification test (NAAT) platforms have been successfully utilized for the detection of arboviruses. In general, the sensitivity of any of the NAATs in identifying arboviruses has been shown to be equal to or greater than the most sensitive viral isolation or antigen detection procedures, while providing equal test specificity. The dynamics of in vivo viral replication and tissue tropisms must be carefully considered so that the utility of a NAAT to a particular arbovirus can be properly applied and interpreted. For example, with encephalitis viruses, the detection of virus in serum using NAAT at the time of clinical presentation is typically unproductive, and a negative result is not informative. Detection of virus in CSF obtained from meningitis or encephalitis patients is often better, with WNV being detected using a real-time reverse transcriptase PCR (RT-PCR) in 14% of acute-phase serum specimens and 57% of CSF specimens (Lanciotti et al., 2000). For nonencephalitic viruses (e.g., DEN viruses) often a much higher viremia with longer duration is achieved, resulting in detectable virus using isolation or NAAT methods. In general, the alphaviruses demonstrate replication kinetics similar to those of the flaviviruses and are not commonly detected in acute-phase serum and/or CSF specimens, although detection is generally greater than with the flaviviruses. In contrast, NAATs have been highly successful in detecting arboviruses from tissues obtained from fatal human cases when the appropriate tissue target is known and assayed (i.e., brain tissue in WNV, LAC, or EEE cases, liver tissue from YF cases, etc.).

RT-PCR

Standard RT-PCR-based assays (compared to real-time assays described below) to detect arbovirus genomic sequences have been developed for a number of agents. These assays use either virus-specific primers or consensus primers that are designed to amplify genetically related viruses. Obtaining a DNA fragment of the predicted size is considered by some to be diagnostic. Greater specificity can be achieved by using sequence-specific approaches for detecting and confirming the identity of the amplified DNA, including hybridization with virus-specific probes (i.e., Southern blot, dot blot, or microtiter plate hybridization), PCR amplification with additional primers internal to the original primers (nested or semi-nested PCR), restriction endonuclease digestion of the DNA product, or nucleic acid sequence analysis. When consensus primers are utilized, a sequence-specific detection method, such as one of those described above, must be employed to specifically identify the resulting DNA, since by the design of the assay, related viruses would all be amplified. Consensus RT-PCR assays have been described for alphaviruses, flaviviruses, and the CAL and Bunyamwera serogroup bunyaviruses (Pfeffer et al., 1997; Kuno, 1998; Lanciotti et al., 1999; Scaramozzino et al., 2001). Virus-specific assays include those for the DEN, YF, Japanese encephalitis (JE), WEE, EEE, SLE, MVE, Powassan (POW), tick-borne encephalitis (TBE), WN, CAL serogroup, Ross River (RR), Ockelbo, and Colorado tick fever (CTF) viruses.

Real-Time 5′ Exonuclease Fluorogenic Assays (TaqMan)

TaqMan RT-PCR assays combine RT-PCR amplification with fluorescent-labeled virus-specific probes able to detect amplified DNA during the amplification reaction. These assays offer numerous advantages over standard RT-PCR, namely, they are quantitative, high throughput, and rapid and have increased sensitivity and specificity. The increased specificity of the TaqMan assay compared to standard RT-PCR is due to the use of the virus-specific internal probe during the amplification. Since postamplification characterization of the amplified DNA is not needed, amplified DNA is not manipulated in the laboratory, resulting in a reduced likelihood of amplicon contamination. Real-time fluorogenic assays also offer the advantage of the ability to detect multiple targets at the same time in the same amplification reaction (multiplexing). Several TaqMan assays for the detection of arboviruses have been described, including those for WN,

SLE, TBE, DEN, EEE, WEE, and LAC viruses (Lanciotti et al., 1992; Kuno et al., 1996; Lanciotti and Kerst, 2001).

NASBA

Another amplification technology which has been used successfully for the detection and identification of arboviruses is nucleic acid sequence-based amplification (NASBA). This approach shares some similarities with RT-PCR at the initial stages; however, there are several significant differences. For NASBA, amplified RNA (not DNA) is detected in a sequence-specific manner. NASBA has been successfully employed for the detection of a number of arboviruses, including WN, SLE, EEE, WEE, LAC, and DEN viruses (Lanciotti and Kerst, 2001).

Advantages and Disadvantages and Tips

Incorporation of MAb reagents into both serological assays and virus identification procedures has led to a new level of test standardization between diagnostic laboratories (Roehrig et al., 1998b). These readily reproducible and highly defined reagents continue to improve the rapidity, sensitivity, and specificity of all diagnostic procedures. Similarly, the exquisite sensitivity of the PCR has created a paradigm shift in how infectious agents are handled and identified. High-containment viruses can be handled safely after they have been subjected to nucleic acid extraction techniques. Since the enhanced sensitivity of the PCR may lead to false-positive results, a diagnosis should not be based solely on a positive PCR result but should be confirmed with a diagnostic serologic assay. Even though PCR and antigen-detection ELISA are rapid and sensitive techniques, it is still useful to actually isolate a virus. Without having a virus in hand, future analyses will be impossible.

While the sensitivity of the newer assays can be spectacular, false positives can still occur. In the MAC-ELISA, the majority of equivocal results occur when *P/N* ratios are between 2.0 and 3.0. In these instances, it is still necessary to confirm these results by an alternative serologic assay. In the antigen-capture ELISA, the inhibition control is often not run, making the capture results uninterpretable (Roehrig et al., 1998b). Similarly, the classical approach of having paired serum samples is also still useful. Even though MAC-ELISA appears to be an excellent way to determine current infections, many times serum samples are taken so early after onset that even the IgM antibody titer is not yet measurable. Both IgM and IgG antibodies will usually be found in convalescent-phase serum samples of these individuals (Roehrig et al., 1998b).

Finally, the best approach to identifying and limiting arbovirus outbreaks is through good disease surveillance. This surveillance may involve sampling of the mosquito vectors or sampling animal reservoirs. Whichever approach is taken, since arboviral diseases know no political boundaries, communication of arboviral activities to agencies like CDC is imperative to formulate a national strategy for disease intervention. CDC is also available to confirm laboratory testing for all labs that have possible arbovirus activity, especially those who have little experience with these diseases and, therefore, utilize commercial laboratories for their arboviral testing. It must be remembered that there is no national certification process for these commercial laboratories, and consequently, some results may not be completely accurate.

Future and Conclusions

There is a continuing need for improvement in laboratory testing for arboviruses. A serologic binding assay to identify the virus specificity of an antiflaviviral antibody response would be quite useful. Even though sequence analysis can identify many unique regions among and between flaviviruses, the conformational dependence of many flavivirus epitopes dictates that sophisticated modeling and structure-function analysis will be needed before these new antigens can be made. New MAbs capable of identifying many arboviruses (especially the medically important bunyaviruses) are also needed. Better and more rigorous testing of new PCR assays is necessary before they can be used routinely in the diagnostic laboratory. Standardization of diagnostic techniques would greatly improve lab-to-lab reproducibility. As new assays are developed, the pharmaceutical industry must take the lead in commercializing these tests and ensuring their validity.

With the exception of human immunodeficiency virus, arboviral infections can be considered the most important emerging or reemerging viral diseases. There are three reasons for this. First, as the world's population continues to grow, humans will continue to encroach on the habitat of these zoonotic viral infections. This encroachment, usually by nonimmune individuals, will result in epidemics of completely new viral diseases or the reemergence of quiescent diseases. Second, as new agents are introduced into the human population, their ability to expand to new areas is facilitated by rapid transportation. An individual infected with DEN virus in Southeast Asia can be back in the United States before symptoms occur. These events could lead to new epidemics in completely new areas, where both physicians and laboratory personnel are unfamiliar with symptoms, diagnosis, and control measures. Finally, there are very few approaches to prevention of these diseases, since neither vaccines nor therapeutic pharmaceuticals exist. With this in mind, the physician and the diagnostic virology laboratory must consider arboviral diseases whenever symptoms, timing, exposure to insects, or travel history indicate possible arboviral infections.

VIRAL PATHOGENS

Introduction

While there are a few exceptions that will be noted later, arboviruses are usually associated with two major disease syndromes—encephalitis or hemorrhagic fevers. Case incidence can vary from hundreds of thousands, as is the case with DEN virus, to a handful, as is the case for the tick-transmitted POW virus, which has caused only 21 reported cases of human encephalitis in the United States and Canada since it was first isolated in 1958. The severity of symptoms of arboviral infections can also vary. Most cases of arboviral encephalitis are subclinical; however, infection with EEE can result in death or severe lifelong neurological deficits. The continental United States has no indigenous arboviruses that cause hemorrhagic fever; however, travelers are at reasonable risk from infection with YF and DEN viruses. Fortunately for the physician, many of the arboviral infections are caused by closely related viruses, so their ecology, entomology, and epidemiology are very similar, regardless of the continent on which the exposure occurred. For example, the recent outbreak of WN encephalitis in Bucharest, Romania, had many features in common with urban outbreaks of SLE in the United States (Tsai et al., 1998; Han et al., 1999). A summary of the biochemical characteristics of the major families of arboviruses is shown in Table 3.

TABLE 3 Characteristics of the families of common arboviruses[a]

Characteristic	Result for virus family:				
	Togaviridae	*Flaviviridae*	*Bunyaviridae*	*Reoviridae*	*Rhabdoviridae*
Size (nm)	60–70	40–60	80–120	60–80	50–100 by 100–400
Morphology	Spherical	Spherical	Spherical	Spherical	Bullet
NA	ssRNA	ssRNA	ssRNA, 3 segments	dsRNA, 12 segments	ssRNA
Polarity of NA	Positive	Positive	Negative	Negative	Negative
Enveloped	Yes	Yes	Yes	No	Yes
Structural proteins	E1, E2, C	E, C, M	L, G1, G2, N, NS_M, NS_S	?	L, G, N, P, M
Nucleocapsid symmetry	Icosahedral	Icosahedral	Helical	Icosahedral	Helical
No. of nucleocapsids	1	1	3	?	1
RNA polymerase	No	No	Yes	Yes	Yes

[a]NA, nucleic acid; ds, double stranded; ?, unknown.

Biology

Alphaviruses

The genus *Alphavirus* in the family *Togaviridae* contains many members that cause disease throughout the world. These viruses can cause classic encephalitis (WEE, EEE, and VEE) or more disseminated disease (Chikungunya [CHIK], o'nyong nyong [ONN], Semliki Forest, Sindbis, RR, Barmah Forest [BF], and Mayaro [MAY]). While these viruses cause a variety of symptoms, their basic biology is identical. An excellent and very comprehensive review of alphavirus molecular biology has been published (Strauss and Strauss, 1994). Alphaviruses are small (60 to 70 nm) viruses with a membrane-derived envelope surrounding an icosahedral nucleocapsid. The nucleocapsid encloses one positive-sense single-stranded RNA (ssRNA) molecule of about 12 kDa. The genome encodes four nonstructural proteins (nsp1 to nsp 4), the capsid (C) protein, and two virus surface glycoproteins, E1 and E2. Little is known about the early events in alphavirus replication. Recent evidence implicates laminin as the alphavirus receptor protein for some cell types (Strauss et al., 1994; Ludwig et al., 1996). Following attachment and endocytosis, the alphavirus must undergo an acid-catalyzed conformational change in its surface glycoproteins that initiates fusion of the viral envelope with the membrane of the endocytic vesicle. This fusion process releases the capsid into the cytoplasm and initiates RNA synthesis. During replication, the structural proteins (C, E1, and E2) are synthesized from a subgenomic mRNA of about one-third of the total genome. This allows for abundant synthesis of the structural proteins for inclusion into progeny virions. Progeny viruses bud through cellular membranes that have been modified by the addition of the E1 and E2 glycoproteins to release infectious virions (Strauss et al., 1995).

Alphaviruses typically kill infected tissue culture cells within 24 to 48 h. Cell death has recently been shown to be through apoptosis (Levine et al., 1994; Ubol et al., 1994; Despres et al., 1995; Levine et al., 1996; Lewis et al., 1996; Griffin and Hardwick, 1997). Alphaviruses are also extremely efficient at shutting down host cell synthesis. Alphaviruses grow well in a number of continuous cell lines, such as Vero, BHK-21, and the mosquito cell line C6/36, any of which are acceptable for virus isolation and subsequent characterization protocols. In fact, by using inoculation procedures and IFA typing with MAbs, alphaviruses can usually be identified in 24 to 48 h.

The E2 protein appears to be the virion protein associated with attachment to susceptible cells. Preincubation of virus with neutralizing anti-E2 monoclonal antibodies alone will block virus attachment to cells (Roehrig et al., 1982; Roehrig et al., 1988; Roehrig and Mathews, 1985). Because of its ability to elicit neutralizing antibody, the E2 protein is under pressure from the immune response, which results in greater sequence divergence than for other alphavirus proteins. It is, therefore, this protein that is most responsible for the specificity of the PRNT in serum from infected individuals. The E1 glycoprotein appears to mediate cell membrane fusion and contains alphavirus group-reactive epitopes (Schmaljohn et al., 1983; Boggs et al., 1989). These E1 epitopes serve as the target for the broadly reactive detector MAbs used in the diagnostic ELISA protocols (MAb 2A2C-3 and 1A4B-6) (Roehrig et al., 1982; Roehrig et al., 1990a; Hunt and Roehrig, 1985).

Flaviviruses

The family *Flaviviridae* is the family of viruses that is responsible for most arboviral disease. This family includes DEN, YF, JE, SLE, and TBE viruses. Other medically important flaviviruses are WN, MVE, and POW. Flaviviruses are small viruses (40 to 60 nm) composed of an icosahedral nucleocapsid surrounded by a membrane-derived envelope. Similar to alphaviruses, the nucleocapsid encloses one positive-sense ssRNA molecule of about 10 to 11 kDa. This genome encodes three structural proteins, capsid (C), premembrane (prM), and E, and seven nonstructural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (Rice et al., 1985). Flavivirus attachment and entry are similar to those of alphaviruses, requiring an acid-catalyzed conformational shift in the E glycoprotein to effect membrane fusion and release of capsid into the cytoplasm (Roehrig et al., 1990b; Guirakhoo et al., 1991; Guirakhoo et al., 1992; Guirakhoo et al., 1993). Unlike alphaviruses, flaviviruses do not have a subgenomic RNA from which the structural proteins are derived. During maturation, the prM protein is cleaved by a furin-like cellular enzyme to M protein, which along with the E glycoprotein, is found in the virion envelope (Stadler et al., 1997). Virus attachment and membrane fusion are both mediated by the E glycoprotein (Guirakhoo et al., 1989; Mandl et al., 1989; Roehrig et al., 1990b). The crystal structure has been solved for the amino-terminal 400-amino-acid fragment of a variety of flaviviruses (Rey et al., 1995; Modis et al., 2003, 2005; Kanai et al., 2006). The three-dimensional structure confirmed much of the biology of the E glycoprotein. For a more extensive review of the flavivirus antigenic structure and function, there are a number of good reviews (Heinz, 1986; Roehrig, 1990; Heinz and Mandl, 1993; Heinz and

Roehrig, 1990). Unlike alphaviruses, flaviviruses do not shut down host cell synthesis. In general, flaviviruses infect a number of continuous cell types but are more selective and take longer to grow. Many flaviviruses may require up to 7 days for adequate antigen expression. Most of the mosquito-borne flaviviruses grow well in the mosquito cell line C6/36.

The E glycoprotein elicits virus-neutralizing antibody, so this protein is subjected to immune pressure and, as a result, is responsible for eliciting virus-specific antibody (Peiris et al., 1982; Kimura-Kuroda and Yasui, 1983; Roehrig et al., 1983; Roehrig et al., 1998a; Hawkes et al., 1988; Barrett et al., 1990). This protein also contains epitopes that are flavivirus cross-reactive (Gentry et al., 1982; Henchal et al., 1982; Roehrig et al., 1983; Roehrig et al., 1998a; Henchal et al., 1985; Hawkes et al., 1988). These cross-reactive epitopes serve as the targets for the broadly reactive detector MAbs used in the diagnostic ELISA protocols (MAb 6B6C-1 and 4G2) (Roehrig et al., 1983; Henchal et al., 1985).

Bunyaviruses

The family *Bunyaviridae* contains the most vector-borne viruses, only a few of which have been consistently associated with human disease. For the United States, the CAL serogroup viruses, primarily LAC encephalitis virus, are the most important pathogens. Other bunyaviruses associated with human disease are the Cache Valley (CV), Jamestown Canyon (JC), Snowshoe hare (SSH), Tahyna, Rift Valley fever, and Inkoo viruses. The bunyaviruses are larger than either alphaviruses or flaviviruses, about 80 to 120 nm in diameter. The virion contains a tripartite genome with three negative-sense ssRNA segments enclosed in helical nucleocapsids surrounded by a lipid envelope (Obijeski et al., 1976b). The L genome segment encodes the l-polymerase, the M genome segment encodes the NS_M protein and the two surface glycoproteins G1 and G2, and the S genome segment encodes the nucleocapsid (N) and NS_S proteins (Obijeski et al., 1976a; Gentsch et al., 1977; Bishop et al., 1980; Bishop et al., 1982). Because of their tripartite genome, there is a potential that bunyaviruses may undergo genetic reassortment in nature, similar to orthomyxoviruses (Gentsch et al., 1977; Bishop et al., 1978; Bishop, 1979; Gentsch et al., 1979; Bishop and Beaty, 1988; Baldridge et al., 1989; Chandler et al., 1990; Chandler et al., 1991; Urquidi and Bishop, 1992).

Coltiviruses

Little is known about the molecular biology of coltiviruses, which are members of the family *Reoviridae*. The virion is naked (60 to 80 nm) and carries 12 negative-sense double-stranded RNA segments within its nucleocapsid (Knudson, 1981). The specific structure of the virion and its structural proteins has not been defined (Attoui et al., 1997; Attoui et al., 1998). The coding assignments are just now being determined, and the functions of the encoded proteins also have not been well defined. Because of the multiple genomic segments, coltiviruses, like bunyaviruses, might be able to undergo genetic reassortment in nature, but this has not been demonstrated to date (Karabatsos et al., 1987).

Rhabdoviruses

Rhabdoviruses are larger viruses (50 to 100 nm by 100 to 400 nm) and have a characteristic bullet shape. Rabies virus is the rhabdovirus of most public health significance; however, the type virus, vesicular stomatitis virus (VSV), has recently been associated with outbreaks in horses and cattle. This virus is included here because of it possible transmission to animal handlers, although such transmission has been rare. Because of its similarities to rabies virus, the molecular biology of the VSV has been intensely studied, and for detailed reviews, the reader is referred elsewhere (Rodriguez et al., 1993; Katz et al., 1997; Letchworth et al., 1999; Alvarado et al., 2002).

Pathogenesis

Arboviruses gain entry through the skin by the bite of an infected arthropod; however, some are capable of being transmitted by aerosol in the laboratory setting. While the knowledge of the initial events of infection is superficial, evidence is accumulating that early interactions of virus, cells, and mosquito saliva might play some role in the outcome of infection (Zeidner et al., 1999). The mosquito saliva enters the dermis and at times enters the small capillaries directly when the mosquito's proboscis penetrates the vessel. It is presumed that the virus replicates initially in the dermal tissues, including the capillary endothelium, although it is also possible that virus is transported directly in the blood to primary target organs. Replication also occurs in the regional lymph nodes, and from there, the blood is seeded, inducing a secondary viremia, which in turn carries virus to infect muscle and connective tissue cells. This viremia is often of very high titer and is accompanied by fever, leukopenia, and malaise. It is during this viremic phase that an arthropod may feed and become infected. The period between infection and viremia (intrinsic incubation period) is usually short, from 1 to 3 days. Viremia may last 2 to 5 days. CTF viremia is of much longer duration because immature erythrocytes are infected and virus remains in the blood cells for 2 to 6 weeks.

The vast majority of human arboviral infections are either asymptomatic or self-limited febrile illnesses. Antibody is produced and it complexes with and neutralizes circulating virus. The process is accompanied by complete recovery and leads to the presence of lifelong antibody. Occasionally, however, an infected person develops encephalitis. The mechanism of entry of virus into the central nervous system (CNS) is not completely understood. Nor is it understood why one person develops encephalitis and another apparently similar individual does not. Virus may reach the brain by seeding of cerebral capillaries during viremia and then by direct invasion of the brain parenchyma through the capillary walls. Alternatively, certain neural cells, such as the olfactory neurons, are exposed directly to circulating blood; viremia may seed these nerve endings and the virus may pass directly to the olfactory lobe of the brain (Monath et al., 1983). Regardless of the mechanism, it is important to note that the process of seeding the brain and productive infection of brain cells takes time. By the time the patient presents with encephalitis, serum antibody is usually detectable, as is antibody, in the CSF. At this stage of infection, viremia has ceased and diagnosis is made by serologic assay.

Epidemiology

Alphaviruses

EEE

EEE occurs throughout the eastern part of the United States. Epidemics of EEE are rare, but a few human cases occur on a regular basis every summer and fall. Equine epizootics also can occur in regions as far north as Canada. The virus is maintained in nature by an enzootic cycle involving birds and a variety of mosquito species. The swampy environments

necessary for the EEE vector mosquitoes usually limit the dissemination of this disease. *Culiseta melanura* is the main mosquito infecting birds; human and equine infections are associated with *Aedes sollicitans* and *Aedes vexans* in temperate regions and with *Aedes taeniorhynchus*, *Culex taeniopus*, and *Culex nigripalpus* in the tropics. A related alphavirus, Highlands J (HJ), also occurs in the eastern United States. While it is not frequently associated with human disease, it can confuse the laboratory diagnosis.

Inapparent cases of EEE are rare. Onset is abrupt with high fever, headache, and vomiting followed by drowsiness, coma, and severe convulsions. On examination, there is neck stiffness, spasticity, and in infants, bulging fontanels. Death may occur within 3 to 5 days of onset. Sequelae are common (30%), including convulsions, paralyses, and mental retardation. The case/fatality ratio for EEE can reach as high as 30%.

WEE

While the last major United States epidemic of WEE occurred in the 1970s, WEE remains an important cause of encephalitis in North America (Reeves, 1987). The enzootic cycle involves passerine birds—in which the infection is inapparent—and culicine mosquitoes, principally *Culex*. Human cases are first seen in June or July in the Northern Hemisphere, but the mechanism of overwintering of the virus is unknown. Children, especially those under 1 year old, are affected more severely than adults and may be left with permanent brain damage, which also is seen in about 5% of adults. The mortality rate is about 25%. Strains of WEE virus appear to be relatively homogeneous by oligonucleotide fingerprinting and are clearly different from the serologically related HJ virus (Trent and Grant, 1980; Hunt and Roehrig, 1985; Karabatsos et al., 1988).

VEE

VEE virus was isolated in Venezuela in 1938 from the brain of a horse; like EEE and WEE viruses, it causes encephalitis in members of the family Equidae and humans. The enzootic cycle of the VEE virus is still incompletely understood but appears to involve a variety of rodents rather than avian species, which are the hosts of the EEE and WEE viruses. Infection of humans is less severe than with the other two alphaviruses, and fatalities are rare. Adults usually develop only an influenza-like illness, and overt encephalitis is usually confined to children. Six antigenic subtypes of VEE viruses (1 to 6) are now recognized, with subtype 1 being subdivided into five varieties, 1AB to 1F (Calisher et al., 1980; Calisher et al., 1985b; Kinney et al., 1983). Only subtype 1AB and 1C viruses have been associated with major epidemics and epizootics (Powers et al., 1997). The other VEE virus subtypes are involved in enzootic VEE virus transmission (Oberste et al., 1996; Watts et al., 1997; Oberste et al., 1998a; Oberste et al., 1998b; Watts et al., 1998).

A major VEE epizootic spread through Central America to reach Texas in 1971, where it was controlled by a massive equine vaccination program, using the live attenuated TC-83 vaccine. Over 200,000 horses died in this outbreak, and there were several thousand human infections. It is now believed that that epidemic was caused by poorly inactivated vaccine (Sneider et al., 1993; Weaver et al., 1999). The most recent outbreak of epizootic VEE occurred in 1995 in Colombia and Venezuela (Weaver et al., 1996; Rivas et al., 1997).

Other Medically Important Alphaviruses

CHIK, ONN, MAY, RR, and BF viruses are exotic alphaviruses with potential for importation into the United States. CHIK, ONN, and MAY are mosquito-transmitted rash diseases. These viruses cause a fairly debilitating acute infection in Africa, Asia, and South America. The disease symptoms usually include headache, fever, rash, and myalgia, but it is not fatal. The mosquito vectors for CHIK (*Aedes aegypti*) and ONN (*Aedes gambiae* and *Anopheles funestus*) are known. The human mosquito vector for MAY has not been well defined, however, *Haemagogus* sp. mosquitoes likely maintain the sylvatic cycle.

RR and BF viruses are the major causes of polyarthritis in Australia. The mosquito vectors are not well defined and vary across Australia. It is believed that the primary vectors are members of the *Aedes*, *Culex*, and *Oclerotatus* genera. Similar to CHIK, ONN, and MAY, acute infection with RR or BF can be debilitating; however, it is not fatal.

Flaviviruses

WNV

WNV was first isolated from the serum of a febrile woman in the West Nile district of Uganda in 1937. Since that time, WNV has circulated in endemic and occasionally epidemic transmission cycles throughout Europe, Western Asia, Africa, the Middle East, Australia (as Kunjin virus), and North and Central America. Major outbreaks of WNV have been documented throughout the world. In 1999, a WNV outbreak was recognized in the United States for the first time. This initial human and animal outbreak was identified in the New York City area. Genetic studies determined that this virus introduction likely occurred from the Middle East, most likely from Israel (Lanciotti et al., 1999). Since that time, WNV has spread westward through the entire continental United States and into Canada, Mexico, Central America, South America, and some Caribbean islands.

WNV now accounts for the largest number of cases of viral encephalitis in the United States. Worldwide, WNV is an arbovirus, primarily transmitted by the mosquitoes of the genus *Culex* (e.g., *Culex tarsalis*, *Culex pipiens pipiens*, *Culex pipiens quinquefasciatus*, *Culex salinarius*, and *Culex nigirpalpus* in the United States). Mosquito-borne transmission to humans in temperate climates usually peaks in the late summer and early fall. Mosquito-borne transmission to humans in milder or more tropical climates can occur throughout the year, whenever mosquitoes are active.

In the United States alone, however, 59 species of mosquitoes have been shown to be infected with WNV. The actual vector status of many of these mosquito species remains to be determined. WNV is a zoonotic disease with birds being the primary natural reservoir. Over 300 species of birds have been shown to be infected with WNV in the Western Hemisphere. Humans are primarily infected through the bite of a WNV-infected mosquito. Recently, other modes of WNV transmission have been identified, such as blood transfusion, tissue transplantation, percutaneous occupational exposure, breastfeeding, and intrauterine transfer (Hayes and O'Leary, 2004). The last two modes of transmission have been documented but are very rare (O'Leary et al., 2006; Paisley et al., 2006).

Even though WNV has now been in the United States since 1999, molecular epidemiological analysis of current and past strains of the United States WNV has demonstrated low-level genetic drift, with remarkable overall phenotypic stability (Lanciotti et al., 1999; Lanciotti et al., 2002; Davis et al., 2005). WNV can be divided into two genetic lineages (1 and 2), with lineage 1 WNVs primarily responsible for major human outbreaks. Lineage 1 strains have been

divided into 4 clades: A, B, Indian, and Kunjin (Beasley et al., 2004).

SLE

SLE virus is also an important mosquito-borne viral cause of epidemic encephalitis in the United States. (Monath and Tsai, 1987; Tsai et al., 1987b; Marfin et al., 1993; McCaig et al., 1994). It also can be found throughout the Western Hemisphere. The last major United States SLE epidemic occurred in the late 1970s, when thousands of individuals in the Midwest were infected. The overall case/fatality ratio is 5% to 15%. Clinical SLE infections have an age-dependent distribution, with the elderly being at highest risk. SLE virus is maintained in nature in a virus-bird-virus cycle. Mosquito vectors in the eastern part of the United States are usually *C. pipiens pipiens* or *C. pipiens quinquefasciatus*. *C. tarsalis* is the primary SLE virus vector in the West. Anecdotal evidence indicates that the eastern form of SLE is symptomatically more severe than the western form. The reasons for this are not known. While SLE is seasonal in temperate areas, year-round transmission can occur in milder climatic areas, such as Florida.

YF

YF is believed to have originated in Africa; the first recorded outbreak was in Barbados in 1647. This was followed by innumerable epidemics in the West Indies, Central and South America, and the eastern United States as far north as New York and by introductions through seaports in more temperate regions in the Western Hemisphere. YF virus was the first virus associated with mosquito transmission and the first flavivirus for which an effective vaccine was developed. Control of the *A. aegypti* mosquito almost completely eradicated urban YF. However, the disease persisted sporadically in rural areas, as a consequence of a sylvatic cycle involving monkeys and forest-dwelling mosquitoes, e.g., *Haemagogus* and *Sabethes* spp. in South America, *Aedes africanus* in East Africa, and a variety of *Aedes* spp. in West Africa.

The disease varies from an inapparent infection to a fulminating disease, terminating in death. After an incubation period of 3 to 6 days, the illness begins suddenly with fever, rigors, headache, and backache. The patient is intensely ill and may suffer from nausea and vomiting. A tendency to bleeding may be seen early on. This stage of active congestion is followed quickly by one of stasis. The facial edema and flushing are replaced by a dusky pallor, the gums become swollen and bleed easily, and there is a pronounced hemorrhagic tendency with black vomit, melena, and ecchymoses. Death, when it occurs, is usually within 6 to 7 days of onset and is rare after 10 days of illness. The jaundice, which gives the disease its name, is generally apparent only in convalescing patients. Mortality can range from 5 to 50%. At autopsy, the organs most affected are the liver, spleen, kidneys, and heart.

DEN

There are four DEN virus serotypes (1 to 4), all of which are endemic throughout the tropics, particularly in Asia, the Caribbean, the Pacific, and some areas of West Africa. DEN is currently the most important arboviral disease, with hundreds of thousands of cases occurring each year and millions of people at risk. In many areas, several types of DEN virus cocirculate, and successive epidemics may occur caused by different serotypes because cross-protection between DEN virus types in humans lasts only a short time.

DEN is endemic in tropical areas where *Stegomyia* spp. mosquitoes are constantly active. *A. aegypti* is the most important vector, particularly in urban areas, but other *Stegomyia* spp. play a role in rural areas of Asia and the Pacific Islands. These include *Aedes albopictus*, *Aedes polynesiensis*, and *Aedes scutellaris*. There is some evidence that monkeys are involved in maintenance of the virus, but there is no proof of a vertebrate maintenance host other than humans.

The clinical picture of classic DEN fever usually affects adults and older children. There is an incubation period of 5 to 8 days, followed by the sudden onset of fever (which is often biphasic), severe headache, chills, and generalized myalgia. A maculopapular rash generally appears on the trunk between the third and fifth day of illness and later spreads to the face and extremities. The illness generally lasts for about 10 days, after which recovery is usually complete, although convalescence may be protracted.

DHF-DSS. In Southeast Asia, DHF-DSS occurs almost entirely in children (Halstead, 1988). More recently, DHF has been reported in Cuba (Guzman et al., 1984; Guzman et al., 1988) and in Brazil (Nogueira et al., 1989); in both of these outbreaks, substantial numbers of adults were affected.

JE

JE is widespread throughout Asia, from the maritime provinces of the former USSR to South India and Sri Lanka (Umenai et al., 1985). Epidemics occur in late summer in temperate regions, but the infection is endemic in many tropical areas. Culicine mosquitoes breeding in rice fields are the main vectors, transmitting virus to humans from water birds and pigs, which act as amplifying hosts. The onset of symptoms is usually sudden and may progress to frank encephalitis. The mortality in most outbreaks is less than 10% but has exceeded 30%.

TBE

TBE is caused by two variants of the same flavivirus: Central European encephalitis (CEE) and Russian Spring and Summer encephalitis (RSSE) viruses. While these two viruses are serologically closely related, the diseases they cause vary in severity. RSSE is the more severe infection, causing a mortality of up to 25% in some outbreaks, whereas that from CEE seldom exceeds 5%. RSSE is transmitted by *Ixodes persulcatus* ticks, whereas *Ixodes ricinus* ticks transmit CEE. CEE can occur in enzootic foci extending from Scandinavia in the north to Greece and Yugoslavia in the south. Males, particularly those who spend large amounts of time in the forests, are at greatest risk from TBE infection. Infection can also be acquired by ingestion of infected cow or goat milk. The infection ranges from mild, influenza-type illness or a benign, aseptic meningitis to fatal meningoencephalitis. Fever is often biphasic, and there may be severe headache and neck rigidity, with transient pareses of the limbs or shoulder girdle or, less commonly, of the respiratory musculature. A few patients are left with residual flaccid paralysis (Ackermann et al., 1986).

POW

POW virus is a rare cause of acute viral CNS disease in Canada and the United States, but it also is present in Russia, where it has been recovered from mosquitoes, ticks, and humans. It was first isolated in Canada in 1958 and has since caused 21 cases of encephalitis in Canada and the eastern United States. Patients who recover may have residual neurological problems. In addition to isolations from humans, the virus has been recovered from ticks (*Ixodes marxi*, *Ixodes cookie*, and *Dermacentor andersoni*) and from the tissues of a skunk (*Spiligale putorius*) (Johnson, 1987).

Bunyaviruses

CAL Serogroup Encephalitis

CAL encephalitis virus was isolated in 1943 from *Aedes* mosquitoes in California and was later associated serologically with three pediatric encephalitis cases in California. Not until 1964, however, was the full significance of the CAL serogroup viruses realized. In that year, a virus closely related to CAL encephalitis virus was isolated from the stored brain of a child who had died in 1960 in La Crosse, Wisconsin. Starting in the early 1960s, the LAC virus has been associated in the United States with about 30 to 140 cases per year of CAL serogroup encephalitis, and LAC encephalitis is currently the most prevalent arboviral encephalitis in the United States. Recently, disease caused by LAC encephalitis virus has been identified in areas outside its classical range of the upper Midwest. LAC encephalitis cases have now been identified in West Virginia, Virginia, Kentucky, Tennessee, North Carolina, and Alabama, indicating either that this virus is emerging into new territory or that it has always been present in these areas and increased surveillance has led to the recognition of disease (McJunkin et al., 1998). Two other closely related CAL serogroup viruses, SSH and JC, have been etiologically associated with a small number of encephalitis cases in the United States and Canada since 1980 (Artsob et al., 1980; Artsob et al., 1982; Grimstad et al., 1982; Artsob et al., 1986).

Most cases of LAC encephalitis are subclinical. Typically, clinical cases of LAC encephalitis occur in children under the age of 15 years. While infection with LAC virus can progress to frank encephalitis, LAC encephalitis is rarely fatal. LAC encephalitis is an endemic disease associated with hardwood forests. The primary mosquito vector is *Aedes triseriatus*, and the virus is maintained in nature in a mosquito-rodent (usually ground squirrels or chipmunks) cycle. An unusual feature of this virus is its ability to be transferred from mother to offspring, by a mechanism known as transovarial transmission in mosquitoes. This mechanism of transmission assists this virus in establishing enzootic foci of infection.

Other Medically Important Vector-Borne Bunyaviruses

Rift Valley fever, Oropouche, Crimean-Congo hemorrhagic fever, Semliki Forest fever, and CV are exotic vector-borne bunyaviruses with potential to be introduced into the United States.

Coltivirus

CTF

CTF is prevalent in the western mountain region of the United States and was initially confused with Rocky Mountain spotted fever until the virus was isolated in 1944 (Florio et al., 1944). There have been 864 confirmed cases of CTF in the United States since 1964, with 551 of these cases occurring in Colorado. It is most common in campers, hikers, and other persons coming in contact with the tick vector *Dermacentor andersoni* (Burgdorfer, 1977; Bowen et al., 1981; Lane et al., 1982; Eads and Smith, 1983; Emmons, 1985, 1988; McLean et al., 1989). Typical symptoms are diphasic fever, muscle aches, malaise, and occasionally, hemorrhagic or CNS complications in children.

Rhabdovirus

VSV

It is not clear whether VSV is an arbovirus in the classical sense, or if insects are purely mechanical vectors of this disease. The main symptoms of VSV in animals are vesicles in the mouth of the infected animals. There is no good evidence that VSV routinely infects humans; and if it does, most of the infections are subclinical. The disease presents as a mild fever. Infection of humans with VSV must be associated with contact with previously infected animals.

Diagnosis

Inclusion of arboviruses as possible etiologic agents of infection in a laboratory differential diagnosis can initially be based on three considerations: case location, time of year the case occurred, and the patient's travel history. A history of mosquito or tick bites is also useful; however, it is usually difficult to document accurately. Domestic arboviral infection has traditionally occurred in the late summer or fall, as numbers of insect vectors increase and enough time has passed for virus amplification in mammalian hosts to allow for transmission to humans. To decide which antigens to use in a lab test for arboviruses, the physician must be aware of any unusual travel prior to the onset of symptoms. If a patient lives in California and has not recently visited the eastern part of the United States, the chance of an EEE infection is nil; however, infection with WEE, WNV, or SLE is a possibility. Similarly, if a patient presents with symptoms consistent with DEN and had traveled to Puerto Rico 6 months earlier, the chance that the current infection is DEN would be small, even though DEN is endemic in Puerto Rico.

Another confounding issue in the diagnosis of encephalitis is the multitude of agents that can cause similar symptoms and the low frequency of arboviral encephalitis. Herpesviruses and enteroviruses also cause encephalitis. Enterovirus encephalitis occurring in the summer months may be confused with arboviral encephalitis. Fortunately, the age distribution of enteroviral encephalitis cases is usually sufficiently different from that of arboviral encephalitides that arboviruses can be ruled out.

The clinical laboratory findings and histopathology of arboviral encephalitis are often not helpful in arriving at an etiologic diagnosis. A definitive diagnosis can be made only in the diagnostic virology laboratory. The histopathology is characterized by perivascular cuffing, neuronal chromatolysis, cell shrinkage, and neuronophagia. EEE brain lesions are unusually necrotizing and are associated with high lymphocyte counts and modestly elevated protein levels in the CSF.

Prevention and Therapy for Arboviruses

The most effective ways to prevent or contain an arboviral outbreak are through vaccination or chemical control of the arthropod vector. Only YF has a currently licensed vaccine that is readily available to the general public in the United States. The YF vaccine is a live, attenuated vaccine that has a long, successful track record and should be recommended for anyone with planned travel into areas of YF endemicity. Both inactivated and live, attenuated vaccines for JE have been developed and successfully used in Asia (Hennessy et al., 1996; Liu et al., 1997). The killed vaccine (Biken, Japan) has been approved for use in the United States A killed vaccine for CEE virus has been in use in Europe for a number of years but is not approved for use in the United States. There are a number of veterinary vaccines available for the equine encephalitides, and there are also experimental vaccines that can be used, with appropriate approval, to protect laboratory personnel from EEE, WEE, and VEE virus infection. These human vaccines, developed by the U.S. Department of Defense, have investigational new drug status and possess

characteristics that will always limit their use in the general public.

Other novel vaccines are being developed for WNV and DEN virus. These newer approaches include DNA and live, attenuated, chimeric viral vaccines. The DNA vaccines express the prM and E proteins of the flavivirus. The chimeric vaccines use the attenuated nonstructural backbones of either YF or DEN viruses combined with the genes for the prM or E proteins of the target flavivirus (Bray and Lai, 1991; Chambers et al., 1999; Chambers et al., 2006; Guirakhoo et al., 2000; Huang et al., 2000; Kochel et al., 2000; Konishi et al., 2000; Monath et al., 2000; Chang et al., 2001; Davis et al., 2001; Huang et al., 2003; Monath et al., 2003; Putnak et al. 2003; Durbin et al., 2006; Guirakhoo et al., 2006; Pletnev et al., 2006; Raviprakash et al., 2006; Simmons et al., 2006; Wu et al., 2006).

Interrupting the virus transmission cycle by reducing human exposure to the arthropod vector remains the most common approach to intervening in an arboviral outbreak. The reduction in human exposure can be accomplished in one of three ways. Since mosquitoes require water in which to breed, source reduction of mosquito breeding sites can reduce the risk of human infection. Source reduction may be either drastic (e.g., draining swamps) or more subtle (e.g., removing items that collect water such as discarded tires or cans). A second and relatively easy approach to reducing human exposure is using insect repellents or reducing time spent outside during the time that mosquitoes are most active. Many of the arboviral mosquito vectors are most active at dusk. Reducing or modifying times of outside activity during the early evening will, therefore, reduce the chance of human infections. The third approach to reducing human exposure is applying either adulticides to reduce the number of biting mosquitoes, or larvicides to reduce future mosquito generations. Either of these techniques is temporary and may require frequent reapplications. As society becomes more sensitized to general application of chemicals, arthropod control through insecticide treatment becomes more difficult. Many of the modern insecticides have been shown to be environmentally and physiologically safe to use and should still be considered where control efforts need to be applied to larger areas. However, with frequent administration of insecticides, mosquito populations can acquire resistance.

As with most viral infections, few therapeutic treatments are available for arboviral infections. Treatment is usually only supportive. A recent study with LAC encephalitis did demonstrate the possible effectiveness of treating patients with Ribavirin (McJunkin et al., 1997). This was the first such study of its kind and will require confirmation in subsequent analyses.

Unusual Features, Insights, Future, and Conclusions

Because of the unique and complex ecology of arboviruses, prevention and control of arboviral disease are difficult. Either control of the mosquito vector or reduction of mosquito breeding sites is usually only a temporary solution. Because of cost, even development of a vaccine as effective as the YF 17D vaccine has not completely controlled this important arboviral disease. What then, are our options to reduce the incidence of these diseases? Vaccine development for those viruses that cause a significant number of infections (e.g., DEN) is finally being pursued by the private sector. The goal will be to produce these products at a cost that will not prohibit their use in resource-poor target populations.

For the "orphan" arboviral diseases (e.g., SLE or EEE) there is little hope that these markets will be lucrative enough for commercial vaccine development. Other approaches must be pursued. One possibility would be the development of protective immune globulin to be used prophylactically in the face of an expanding epidemic. Progress in producing human MAbs or humanization of protective murine MAbs may lead to a cheap source of readily available products capable of aborting viral infection. In animal models, it appears that preexisting neutralizing antibody may be sufficient to abort infection or, in some circumstances, cure infection (Mathews and Roehrig, 1982, 1984; Roehrig and Mathews, 1985; Boere et al., 1983; Schlesinger et al., 1985; Kaufman et al., 1987; Oliphant et al., 2005; Morrey et al., 2006; Morrey et al., 2007). What is known is that through adequate disease surveillance, epidemics could be detected very early, and the at-risk population could be identified and protected by these reagents.

As with most viral infections, it is not easy to produce therapeutic agents. The use of ribavirin for LAC infections appears to be promising. Since most medically important arboviruses are positive-stranded RNA viruses, there are few such compounds available. As with vaccines, because the potential market for these therapeutic agents may be small, it will be difficult to entice the pharmaceutical companies to develop therapeutics specifically for arboviruses. For flaviviruses, research and development on therapeutic drugs for hepatitis C may be directly applicable to the mosquito- and tick-borne members of this family. It is clear, however, that therapeutic drugs for most of these viruses are not on the immediate horizon. Because of these factors, arboviral diseases will continue to affect mankind for the foreseeable future.

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Human Papillomaviruses

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Papillomaviruses are small, nonenveloped, icosahedral viruses that have a double-stranded DNA genome and that multiply in the nucleus. They are widely distributed in nature and infect humans, monkeys, cattle, rabbits, dogs, and many other species (Fig. 1). Human papillomaviruses (HPVs) infect surface epithelia and produce warts or other pathology at the site of multiplication on the skin or the mucous membrane. HPVs are etiologically associated with benign tumors (cutaneous and genital warts and respiratory papillomas) as well as with cancer of the uterine cervix and of other genital tract sites. They also are responsible for a subset of cancers of the oropharynx, especially tonsillar cancer. HPV-based prophylactic vaccines introduced in 2006 show great promise for future reduction in the burden of cervical cancer, a major cancer of women in the developing world.

The infectious nature of human warts was established in 1907 by experimental transmission of warts from person to person by inoculation of a cell-free extract of wart tissue. The virus was visualized in the 1950s soon after electron microscopy came into general use. Warts have characteristic histopathological features and have been recognized at many different sites in humans (skin, genital tract, respiratory tract, and oral cavity) and in many mammalian species. However, HPVs still cannot be grown in conventional cell cultures. The existence of a large number of distinct HPVs became evident only after the development of recombinant DNA technology, which permitted the cloning of viral genomes from different sites and the comparison of the nucleotide sequences of these genomes. To date, over 150 different HPV types have been recognized.

CHARACTERISTICS OF THE VIRUS

Papillomaviruses are classified on the basis of species of origin (human, bovine, etc.) and the degree of genetic relatedness with other papillomaviruses infecting the same species. New types are defined by the extent of sequence variation from known types in specific regions of the genome (de Villiers et al., 2004). The virion is nonenveloped and has a diameter of 55 nm, icosahedral symmetry, and 72 capsomers. The viral genome is a double-stranded, circular DNA molecule with 8×10^3 bp and a molecular mass of 5.2×10^6 Da. Complete nucleotide sequences are known for many HPV types. All of the open reading frames (ORFs) in papillomavirus DNA are located on only one of the two strands, indicating that only one strand carries the genetic information. Detailed physical maps have been constructed for almost all of the HPV genomes. The viral genome is divided into an early region that contains eight ORFs (E1 to E8) and a late region that has two ORFs (L1 and L2).

The functions of the papillomavirus ORFs are listed in Table 1. The viral capsid is made up of two structural proteins, L1 and L2. L1 is the major structural protein of the virus. It mediates viral attachment to susceptible cells and the immunologic responses to viral infection. The L1 protein produced in yeast or in a baculovirus vector self-assembles as a virus-like particle (VLP) that is conformationally similar to the authentic virion (Schiller and Lowy, 1996). The commercially available HPV-based preventive vaccines employ L1 VLPs as the immunogens.

The early proteins E1 and E2 are viral regulatory proteins involved in viral DNA replication and viral transcription. E4 is a late protein expressed in terminally differentiated cells and is found in association with viral capsids. E6 and E7 are the oncoproteins of HPVs that are responsible for the immortalization and transformation of keratinocytes. The E6 and E7 proteins exert their effects, in part, by complexing with and inactivating, respectively, the tumor suppressor proteins p53 and pRb. The E6 and E7 proteins are invariably expressed in cells of HPV-associated cervical cancer and are the targets for HPV-based immunotherapy protocols, which aim to destroy established cervical cancers (Wu, 1994).

PATHOGENESIS

HPVs infect only epithelia of skin and mucous membranes. The virus probably infects cells of the basal layer of the epithelium, which undergo proliferation and form the wart. Histologically, a wart is a localized epithelial hyperplasia with a defined boundary and an intact basement membrane. All layers of the normal epithelium are represented in the wart. The prickle cell layer is irregularly thickened, the granular layer contains foci of koilocytotic cells, and the cornified layer displays hyperkeratosis. The viral capsid antigen and virus particles are found only in the nuclei of cells of the differentiated, nondividing, superficial layers of the wart. In the infected cell, the multiple copies of the viral genome are present in an unintegrated state.

Warts and other papillomavirus-related lesions vary widely in appearance, morphology, site of occurrence, and patho-

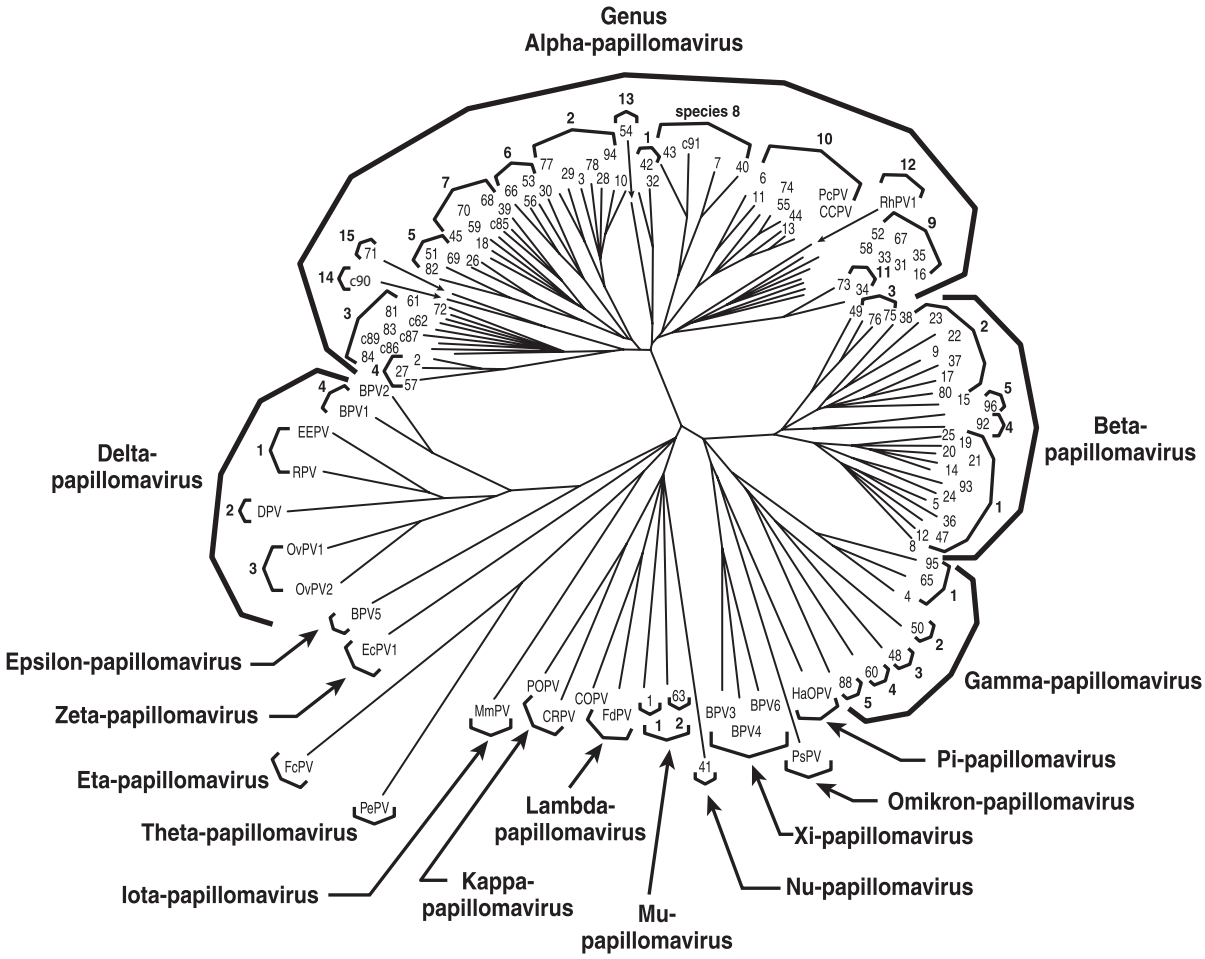

FIGURE 1 Phylogenetic tree containing the L1 open reading frame sequences of 118 papillomavirus types. The numbers at the ends of each of the branches identify HPV types; c-numbers refer to candidate HPV types. All other abbreviations refer to animal papillomavirus types. The outermost bracketed symbols identify papillomavirus genera, e.g., the genus alpha-papillomavirus, beta-papillomavirus, etc. The inner brackets and corresponding numbers refer to species within the individual genus. For example, the upper part of the figure shows that HPV types 7, 40, 43, and c91 together form the HPV species 8 in the genus alpha-papillomavirus. (Adapted from de Villiers et al., 2004.)

genic potential. The spectrum of HPV infection ranges from completely subclinical infection, to transient, barely noticed, self-limiting, benign infections, to malignancies of the skin or the genital tract. Many factors determine the clinical significance of papillomavirus infection, as described below.

Location of the Lesion

The importance of the location of the lesion is best exemplified by laryngeal papilloma. Although the tumors are benign, they may cause life-threatening respiratory obstruction because of their location on the vocal cords.

Genotype of the Virus

There is a strong correlation between the genotype of the infecting virus and the morphology and site of the lesion. For example, almost all flat warts of the skin yield HPV type 3 (HPV-3) or HPV-10. Most deep plantar warts are caused by HPV-1, and common warts are caused by HPV-2. Virus types HPV-6 and HPV-11 are recovered from most of the genital warts (condylomas). Oncogenic potential is also correlated with the viral genotype. In the genital tract, HPV-16 and HPV-18 are strongly associated with malignancies, and HPV-6 and HPV-11 are associated with benign warts. In the rare dermatologic disorder epidermodysplasia verruciformis (EV), lesions caused by HPV-5, HPV-8, and HPV-13 have a greater tendency to convert to malignancy than lesions caused by several other virus types.

Host Factors

Warts tend to increase in size and numbers in conditions associated with immunologic impairment, especially T-lymphocyte deficiency. The immunologic impairment may be subtle, as in pregnancy, or gross, as in organ transplant recipients, patients receiving anticancer therapy, and AIDS patients.

Papillomavirus infection is acquired in a variety of ways: through skin abrasions (skin warts), by sexual intercourse (genital warts), during passage through an infected birth canal (juvenile-onset laryngeal papilloma), and probably in other ways (e.g., papillomas of the oral cavity by autoinoculation or by oral sex).

TABLE 1 Papillomavirus ORFs and their functions and products

ORF[a]	Function or product
L1	L1 protein is the major structural protein; proposed immunogen for preventive vaccines
L2	L2 protein is a minor capsid protein
El	Initiation of viral DNA replication
E2	Regulation of viral transcription
E4	Expressed late; disrupts cytokeratin and aids in virus release
E5	Interacts with growth factors; oncogenic for bovine papillomavirus
E6	Transforming protein; targets p53 degradation
E7	Transforming protein; complexes with retinoblastoma protein

[a]ORFs E3 and E8 have no known functions.

DISEASE POTENTIAL

The HPVs naturally fall into two groups, cutaneous HPVs and mucosal HPVs. The viruses are site specific. Cutaneous HPVs are seldom encountered in the genital tract, and genital HPVs are rarely found on the skin. The reservoir for all of the mucosal HPVs is the genital tract, with two exceptions. HPV-13 and HPV-32 are viruses of the oral cavity associated with a condition called focal epithelial hyperplasia, which is prevalent largely in some aboriginal populations. Genital HPVs are also recovered from other mucosal sites, especially the aerodigestive tract. HPV-6 and HPV-11, which are responsible for condylomas in the genital tract, may be transmitted intrapartum from an infected mother to the child and produce juvenile-onset recurrent respiratory papillomatosis (laryngeal papilloma). The HPV-associated illnesses and the most common types of virus responsible for these conditions are listed in Table 2.

Cutaneous HPVs

Cutaneous Warts

There are many morphological types of warts, and each type may have preferred locations on the skin (Bunney, 1992). Specific HPV types are associated with different morphological types of lesions (Croissant et al., 1985). Common warts (caused by HPV-2 and HPV-4) are found on the hands and generally occur as multiple warts. The warts are characteristically dome shaped, with numerous conical projections (papillomatosis) that give their surfaces a velvety appearance. Deep plantar warts (on the bottom surface of the foot; caused by HPV-1) generally occur singly and have a highly thickened corneal layer (hyperkeratosis). Flat warts (with little or no papillomatosis; caused by HPV-3 and HPV-10) almost always occur as multiple warts and are found most often on the arms and face and around the knees. The threadlike filiform warts occur most often on the face and neck.

Skin warts are transmitted by direct contact with an infected individual or indirectly by contact with contaminated objects. The incubation period is difficult to estimate but may be as short as 1 week or as long as several months. As a rule, warts in an otherwise healthy individual are few and small, but a large number of warts may develop in immunodeficient individuals or in apparently healthy persons. Most warts regress within 2 years, probably as a result of cell-mediated immune responses. Treatment or excision of one wart often results in regression of the remaining warts. This may result from a "triggering" of an immune response due to immune-competent cells which come in contact with antigens that are released as a result of treatment.

Warts are most prevalent in children and young adults. At any time, as many as 10% of school children may have warts at some site. It is not known if the reduced prevalence in the older population represents acquired immunity, reduced exposure, or both. The incidence of warts in the general population is believed to be increasing. Recreational activity in which bare skin may be exposed to virus-contaminated objects (for example, swimming in communally used pools) increases the risk of acquiring warts, especially plantar warts (Bunney, 1992).

EV

EV is a rare, lifelong disease in which a patient is unable to resolve the wart virus infection (Jablonska and Majewski, 1972). Most patients exhibit defects of cell-mediated immunity. The disease probably has a genetic basis. Patients frequently give a history of parental consanguinity, and despite the rarity of the disease, multiple cases occur in some families. It is postulated that EV patients have an inherited immunologic defect as a result of homozygosity for a rare recessive autosomal gene. A susceptibility locus was recently mapped to chromosome 17q25, and truncating mutations in either of two novel adjacent genes, EVER1 and EVER2, are associated with the disease in different pedigrees (Ramoz et al., 2002). The function of the gene products of EVER1 and EVER2 and how they confer increased risk for EV are unknown.

The onset of EV occurs in infancy or childhood. The patient develops multiple, disseminated, polymorphic wart-like lesions that tend to become confluent. The warts are of two clinical types: flat warts and red or reddish-brown macular plaques resembling pityriasis versicolor. The warts contain abundant amounts of virus particles, viral antigen, and viral DNA. The flat warts of EV patients yield HPV-3 or -10, the same genotypes that are recovered from flat warts of healthy individuals. However, a bewildering variety of viral genotypes are recovered from the macular plaques of EV patients (Jablonska and Majewski, 1972). These EV genotypes have not been recovered from skin warts, so it is not clear how EV patients acquire these infections. Recent reports suggest that these EV-related viruses are widely seeded in normal skin epithelium (Boxman et al., 1997).

In about 33% of the cases, multiple foci of malignant transformation arise in the reddish-brown plaques, especially in lesions occurring in areas exposed to sunlight. Histologically, the tumors may be in situ (bowenoid) or invasive squamous cell carcinomas. The tumors grow slowly and are generally nonmetastasizing. The malignant cells contain multiple copies of episomal viral DNA (HPV-5, -8, or -14) but no viral particles or capsid antigen. HPV DNA is also recovered from metastatic tumor cells.

The carcinomas occurring in EV patients illustrate how several factors working in concert result in papillomavirus-induced malignancy. Viruses of specific genotypes infecting an immunologically impaired host produce malignant transformation in lesions that are exposed to sunlight.

Skin Cancers

DNAs of numerous HPV types, many of them novel types, have been recovered from nonmelanoma skin cancers in renal transplant recipients as well as in healthy individuals, but it is not clear to what extent they contribute to the development of these cancers (Pfister and ter Schegget, 1997).

TABLE 2 Clinical associations of HPVs

Disease location and type	Predominant HPV type(s)
Skin	
Deep plantar wart	HPV-1
Common wart	HPV-2, -4
Flat wart	HPV-3, -10
EV macular plaques	HPV-5, -8, etc.
Mucosa	
Genital warts	HPV-6, -11
Cervical cancer	
High risk	HPV-16, -18, -31, -45, -33, -35, -39, -51, -52, -56, -58, -59, -68
Low risk	HPV-6, -11, -42, -43, -44, etc.
Vulvar cancer	HPV-16
Penile cancer	HPV-16
Oropharyngeal cancer	HPV-16
Respiratory papillomas	HPV-6, -11
Focal epithelial hyperplasia of the oral cavity	HPV-13, -32

In both skin cancers and normal skin, the HPV DNA prevalence is high, multiple types are common, and the amount of DNA is very low, indicating that the DNA is present in a small fraction of the tumor or normal cells (Berkhout et al., 2000; de Koning et al., 2007). EV-related HPV types constitute the most frequent types, but mucosal HPVs also are reported from skin lesions. It has been difficult to demonstrate viral transcripts in the DNA-positive tissues or to associate specific HPV types with the cancers. It is unlikely that the cutaneous HPVs are associated with skin cancers in the same way as genital HPVs are associated with cervical cancer.

Mucosal HPVs

About 40 HPV types infect the genital tract. Genital HPV infections are the most prevalent sexually transmitted infections, with prevalence as high as 40 to 45% in sexually active young women (Schneider and Koutsky, 1992). A large majority of the HPV-positive women have no cytological abnormalities. The infections have a duration of 6 to 24 months. The prevalence decreases markedly with increasing age.

Genital Warts (Anogenital Warts, Condyloma, and Genital Papilloma)

Papillomavirus infection of the genital tract occurs predominantly in young adults and in sexually promiscuous populations. Genital warts are the most frequent clinical manifestation of HPV infections. The incidence of genital warts has increased in the United States since the end of the decade of the 1960s. In 2007, the number of individuals consulting physicians for genital warts was 312,000, compared to 56,000 in 1966 (Centers for Disease Control and Prevention, 2007). The number of annual visits to physicians for genital warts, reported to the CDC, remained around 200,000 throughout the 1990s. However, in 2005, the rate increased to 357,000. In the United Kingdom, the annual incidence of genital warts per 100,000 population rose from about 30 in 1971 to 50 in 1978. In sexually transmitted disease clinics, genital warts account for about 4% of patient visits, compared with 24% of visits accounted for by gonorrhea; however, in a population-based study in Rochester, MN, the incidence rate for genital warts was about one-half that for gonorrhea (Chuang et al., 1984).

The incubation period for condylomas is estimated to be between 3 weeks and 8 months, with an average of 2.8 months (Oriel, 1971). About 66% of the sexual partners of condyloma patients develop the disease. Condylomas may be papillary (condyloma acuminatum) or flat (condyloma planum). The most frequent sites for papillary (or exophytic) condylomas are the penis, around the anus, and on the perineum in the male and the vaginal introitus, the vulva, the perineum, and around the anus in the female. On the cervix, flat condylomas are far more frequent than papillary condylomas (Meisels et al., 1982). The flat lesion on the cervix was not recognized to be due to papillomavirus infection until the late 1970s. It is now known to be a common clinical manifestation of genital HPV infection in the female. The lesion is generally seen only by a colposcopic examination and is confirmed by cytology and histopathology.

In a large number of infected individuals, condylomas occur at more than one site in the genital tract. Condylomas may increase in number and size during pregnancy and regress after delivery. Immunosuppressed populations—for example, patients with AIDS—have a high prevalence of condylomas. The closely related HPV-6 and -11 are responsible for a large majority of the condylomas (Gissmann et al., 1983). Many genital warts regress with time, but some may persist for long periods. They may cause local irritation and itching, become infected, and cause severe physical and psychological difficulties for the patient if they enlarge in size or increase in numbers. The presence of condylomas during pregnancy is a risk factor for the transmission of HPV from mother to newborn during birth and for the consequent development of respiratory papilloma in the offspring.

Recurrent Respiratory Papillomatosis

Recurrent respiratory papillomatosis is a chronic, rare, and recurrent disease in which benign viral papillomas in the respiratory tract may become life-threatening because of their location. The vocal cords in the larynx are the site most often affected, although the disease may occur at other locations (e.g., the trachea) without laryngeal involvement. The most common presenting symptom is hoarseness of voice or change of voice. The papillomas may produce respiratory distress and obstruction, especially in children. The disease tends to recur following surgical removal of the papilloma, and patients may require frequent operations, sometimes as often as every 2 to 4 weeks. Surgery may lead to dissemination of disease to other sites, for example, to the lungs. Malignant conversion of papilloma is rare; it may be associated with a history of previous radiation therapy but may also occur in the course of a long-term chronic papillomatosis.

The highest risk of onset of respiratory papilloma is under the age of 5 years. About 33 to 50% of the cases occur by that age, and the onset of illness in about 33% of the cases occurs in adult life (Mounts and Shah, 1984). The viral types recovered from both juvenile- and adult-onset disease are HPV-6 and -11, the viruses that are responsible for genital warts. The transmission of virus in juvenile-onset cases occurs during the process of birth in the course of fetal passage through an infected birth canal. Mothers of patients with laryngeal papilloma frequently have a history of genital warts during pregnancy. In a population-based study in Denmark, a history of condyloma during pregnancy was shown to increase the risk of respiratory papilloma in the child by 200-fold; the risk of acquiring laryngeal papilloma for children born to

mothers with active genital papillomavirus infection was estimated to be less than 1 in 100 (Silverberg et al., 2003). Cesarean delivery prior to rupture of the membrane very likely reduces the risk of virus transmission. The transmission of virus in adult-onset disease does not occur intrapartum. It is suspected that in adults, the infection is acquired by oral contact with infected genitalia.

Warts at Other Mucosal Sites

Several morphological types of warts occur in the oral cavity. They have been described as common warts, flat warts, condylomas, or respiratory papillomas on the basis of their clinical and histologic features. HPV-6 and HPV-11 are the viruses recovered most frequently from these lesions. A clinically well-defined entity, focal epithelial hyperplasia, has been described in the oral mucosa. The condition occurs with high frequency in American Indians in North and South America, but it also has been seen in other races and in many parts of the world. Clinically, there are discrete, multiple, elevated nodules on the oral mucosa (lips, buccal mucosa, and tongue), which may persist for many years and have the histologic appearance of warts. These lesions are associated with HPV-13 and -32 (Pfister et al., 1983).

Cervical Cancer

Worldwide, about 500,000 new cases of cervical cancer occur each year. The incidence of cervical cancer is high in developing countries, where it is the most common female malignancy and accounts for about 24% of all female cancers. In the United States, there are about 14,000 new cases of cervical cancer and about 4,800 deaths annually.

Nearly all cervical cancers originate in the squamocolumnar transformation zone of the cervix, where columnar cells from the endocervix form a junction with the stratified epithelium of the vagina. A progressive spectrum of abnormalities, classified as low-grade and high-grade squamous intraepithelial neoplasia, precedes invasive cancer. Most of these abnormalities resolve on their own, and only a small proportion progress to invasive cancer. The time interval between early cytological abnormalities and cervical cancer may span 10 to 20 years. During this interval, cytological abnormalities are readily detected by Pap smear examination and can be successfully treated.

The evidence for an etiologic link between HPV infections and cervical cancer is conclusive (Bosch et al., 2002). Almost all cervical cancers in all parts of the world are caused by HPV infections (Bosch et al., 1995; Walboomers et al., 1999). This evidence is briefly summarized below.

Cancer of the cervix has the epidemiological characteristics of a sexually transmitted disease. The wide differences in cervical cancer incidence in the world are fully explained by taking into account HPV endemicity, sexual practices of women and of their male partners, and standards of healthcare, i.e., access to Pap smear screening and to treatment of preinvasive disease (Skegg et al., 1982; Bosch et al., 1994).

HPV DNA sequences are found in a large majority of invasive cancers as well as in the entire clinical spectrum of precursor lesions of invasive cancers. There is a preferential association of some HPV types with invasive cancer (Table 2; Fig. 2). While all HPV types found in the genital tract are associated with some form of mild dysplasia and/or subclinical infections, HPV-16, HPV-18, HPV-31, and HPV-45 predominate in invasive cancers. The viral genome is found in the cancer cells themselves and is present in both the primary and metastatic tumors. The viral genome is extrachromosomal in most dysplasias but is integrated in all HPV-18-associated and many HPV-16-associated invasive cancers (Cullen et al., 1991). The linearization of the viral genome prior to integration occurs most frequently by a break in the E1-E2 region, leading to the deregulation and increased transcription of the viral genes E6 and E7. The E6 and E7 genes, which code for the transforming functions of HPV, are always expressed in HPV-associated cancers. Consequently, antibodies to E6 and E7 proteins are frequently found in patients with HPV-associated invasive cancers but are rare in healthy individuals. Experimental data indicate that the E6 and E7 proteins of the high-risk HPVs exert their oncogenic effect by complexing with and inactivating the tumor suppressor proteins p53 and pRb105, respectively (Scheffner et al., 1991). Mutations of the cellular p53 and pRb are rare in HPV-associated cancers because these tumor suppressor proteins are inactivated by the viral oncoproteins.

HPV-16 is the virus most predominantly associated with cervical cancers in worldwide studies and accounts for about 50% of the cancers. HPV-18 is associated with 10 to 20% of cervical cancers and is not distributed uniformly in different geographic areas. HPV-16 and related viruses (HPV-31, -33, -35, -52, and -58) and HPV-18 and related viruses (HPV-39, -45, -59, and -68) together account for more than 90% of invasive cervical cancers. HPV-18 and HPV-16 infections account for about equal proportions of adenocarcinoma of the cervix.

Cancers at Other Genital Sites

HPV infections are associated with subsets of cancers at other genital sites. It is estimated that about one-half of vulvar, vaginal, and penile cancers and about 90% of cancers of the anal canal are attributable to HPV infections (Parkin and Bray, 2006). HPV-associated vulvar cancers have a basaloid pathology, occur in younger women, and have sexual history risk factors.

Oropharyngeal and Tonsillar Cancer

A large majority of the 550,000 head and neck cancers that occur annually worldwide are associated with alcohol and tobacco use, but a subset of these cancers is etiologically linked to HPVs (D'Souza et al., 2007). The HPV-caused cancers are located almost exclusively in the oropharynx, where they constitute a majority of cancers arising from the lingual and palatine tonsils. They are defined by the presence of a transcriptionally active HPV genome localized to the nuclei of the tumor cells. The HPV-associated tumor has basaloid pathology and rarely has mutations of the cellular tumor suppressor proteins p53 and pRb. A history of multiple sexual partners and of oral sex, rather than of alcohol and tobacco use, are risk factors for an HPV-associated tumor. Patients with HPV-associated oropharyngeal cancers have a better prognosis than patients with non-HPV-associated oropharyngeal cancers. HPV-16 accounts for more than 90% of the HPV-positive tumors.

DIAGNOSIS

Clinically, a papillary wart is seldom misdiagnosed as something else, but other dermatologic conditions (e.g., molluscum contagiosum, plantar corn, or skin tags) may be mistaken for warts. Histologic examination of the tissue generally establishes the diagnosis of a wart but does not assist in identification of the genotype of the infecting virus. No serologic tests are available for virus identification.

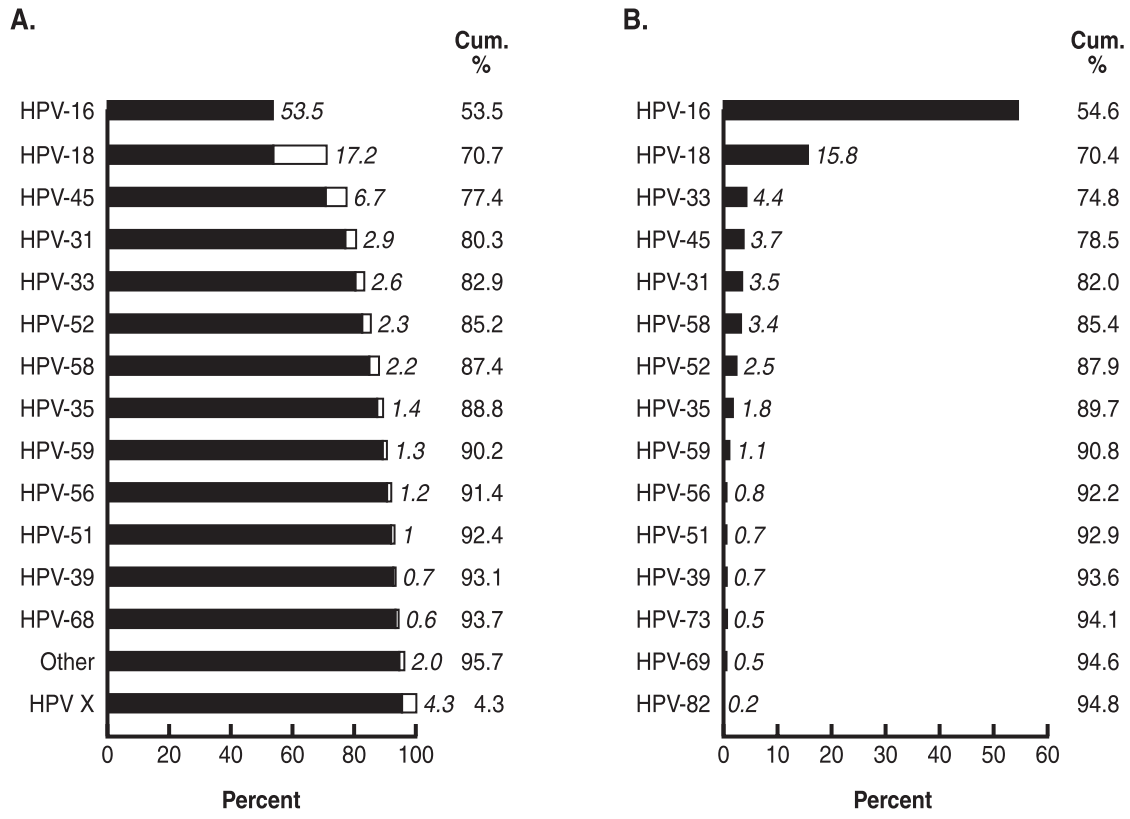

FIGURE 2 Percentages of cervical cancer cases attributed to the most frequent HPV types in all world regions combined, as estimated from pooled analysis of 3,085 cases reported to the International Agency for Research on Cancer (A) and meta-analysis of more than 14,500 cases reported in the literature (B). (Adapted from Clifford et al. 2006.)

Tests for Viral Capsid Antigen

A broadly cross-reactive genus-specific antiserum is available that is capable of recognizing capsid antigen of all HPVs and animal papillomaviruses by immunoperoxidase or immunofluorescence tests (Jenson et al., 1980). Tests can be performed on sections of routinely collected, formalin-fixed, paraffin-processed tissues as well as on exfoliated cells. The viral antigen is present in the nuclei of cells of the superficial layers of the epithelium. For detection of virus, an immunologic test for viral capsid antigen is considerably more sensitive than demonstration of virus particles by electron microscopy. However, the antigen is not detectable in at least 25% of histologically confirmed warts. In antigen-positive tissues, the number of cells displaying antigen is variable, ranging from only one or two cells to a large number of cells in the section. Only a proportion of cytologically affected cells exhibit antigen. Warts at different sites differ markedly with respect to their yield of virus particles and patterns of antigen distribution. Virus particles and antigen are abundant in some plantar and common warts but are scarce in genital tract and laryngeal papillomas.

The capsid antigen assay has several limitations. Productive infection ceases as the lesion progresses toward malignancy, so the assay is negative in HPV-associated cancers. Also, the assay, when positive, indicates that there is productive infection with an HPV but does not identify the genotype of the infecting HPV.

HPV Identification by DNA-Based Assays

HPVs can be specifically identified only by nucleic acid-based assays because the viruses cannot be grown in culture and type-specific immunologic reagents are not available. Tests in common use are (i) PCR-based assays in which a segment of the HPV genome is first amplified and then identified by hybridization, by sequencing, or by its restriction fragment pattern; (ii) hybridization of unamplified tissue DNA with viral probes by hybrid capture or by Southern hybridization; and (iii) in situ hybridization of tissue sections to localize the viral genome to specific cells.

The PCR assays are most frequently used for epidemiological investigations. Hybrid capture is used for both epidemiological and clinical studies. The in situ hybridization assay is most useful in studies in which it is important to localize the virus to the tumor cells.

PCR-Based Assays

PCR-based assays have high analytic sensitivity and can detect as few as 10 to 100 copies of the HPV genome. They require very small amounts of the specimen. The most widely used assays to identify genital HPVs employ a one-step treatment of the clinical specimens and a single amplification reaction with consensus primers, which are capable of amplifying most of the genital tract HPVs. The PCR products are then identified with a large number of type-specific probes, by sequencing the PCR products, or by an analysis of the restriction fragment patterns of the PCR products.

The two most widely used PCR assays both amplify a segment of the L1 ORF. The MY09-MY11-HMB01 primers are degenerate, and they amplify a 450-bp segment of the L1 ORF of HPVs (Manos et al., 1989). The PCR products are identified in a dot blot format by hybridization with individual biotinylated type-specific HPV probes employing Amersham's enhanced chemiluminescence system. A simpler variation of this assay has been published recently in which amplification is performed with biotinylated primers and the PCR products are identified by reaction with immobilized probes on a strip (Gravitt et al., 1998). This line blot assay requires a single hybridization assay. The GP5+ and GP6+ primers, which are also widely used, are consensus, nondegenerate primers which amplify a 140-bp region of the L1 gene (de Roda et al., 1994).

Hybrid Capture Assays

The Hybrid capture assay (Digene, Inc., Beltsville, MD) is performed in a 96-well microtiter format on unamplified tissue DNA (Lorincz, 1996). The sensitivity of the assay is enhanced by signal amplification. The tissue DNAs are screened separately with each of two pools of full-length RNA probes: probe A consists of the low-risk HPV types HPV-6, -11, -41, -42, and -43, and probe B consists of the 13 high-risk and intermediate-risk HPVs listed in Table 2. The single-stranded DNA in denatured specimens hybridizes with the RNA probe. The DNA-RNA hybrids are captured and immobilized with a hybrid-specific antibody on the bottom of the well and detected in an enzyme-linked immunosorbent assay-like format with the use of a chemiluminescent compound. The virus in the specimen is identified as belonging to a low-risk or high-risk group but is not identified as a specific type. The test allows for a degree of quantitation of the viral DNA in the specimens. The Hybrid Capture II system is available commercially and is approved by the U.S. Food and Drug Administration.

In Situ Hybridization

In situ hybridization is the only method that permits localization of the viral genome to a specific cell type, e.g., tumor cells. Tests have been devised to detect viral DNA, viral transcripts, or expression of specific viral genes (Durst et al., 1992).

HPV Serology

Recently, type-specific serological assays for HPV that use recombinant capsid proteins as antigens have been developed (Kirnbauer et al., 1994). The assays are primarily useful for population-based studies to determine rates of exposure to different HPV types and for epidemiological studies to establish associations of HPV exposure with disease. The tests are not useful for diagnosis of acute infection because the sensitivity is only ~50% compared to HPV DNA detection methods, and the tests cannot distinguish current infection from past exposure (Dillner, 1999). Serological assays for HPV E6 and E7 antibodies have a sensitivity of 40 to 60% and a specificity of >90% for HPV-associated invasive cancer (Viscidi et al., 1993). However, antibodies to E6 and E7 are rarely detected in individuals with preinvasive lesions, and thus, the assays are not useful for cancer screening (Lehtinen et al., 2003). In invasive cervical cancer, antibody responses to E6 and E7 do not have prognostic value and are not useful for monitoring response to therapy (Silins et al., 2002).

TREATMENT

Treatment of Warts

Most skin warts and genital warts regress spontaneously. The patient seeks treatment for cosmetic reasons, pain, discomfort, and disability depending on the location and size of the warts. The most difficult problems for therapy are posed by children with recurrent laryngeal papilloma, patients with EV, pregnant women with genital warts, and warts in immunocompromised individuals. There is no "one-time" treatment for all warts. The therapies in use include application of caustic agents, such as podophyllin and salicylic acid; cryotherapy; surgical removal; antimetabolites, such as 5-fluorouracil applied in a cream or a solution; 5% imiquimod cream; and treatment with interferon. Both laryngeal papillomas and genital warts are reported to respond to interferon therapy, but recurrence after cessation of therapy is not uncommon. Preinvasive cervical lesions are readily treated by a variety of procedures (cryotherapy, laser, loop electrocautery excision procedure, etc.).

Prophylactic HPV-Based Vaccines

In the early 1990s, as the causative role of HPVs in cervical cancer appeared to be very likely, the efforts to develop a preventive HPV-based vaccine against cervical cancer gathered speed. Immunization-challenge studies in dogs, rabbits, and cattle with the respective animal papillomaviruses indicated that a vaccine based on the major viral protein (L1) would be protective against homologous viral challenge. It was shown that the L1 coat protein expressed as a recombinant protein could self-assemble into a VLP and that human immunization with VLPs resulted in a high-titered type-specific antibody response. A "proof of concept" trial (Koutsky et al., 2002) with HPV-16 VLPs showed that three doses of the vaccine provided nearly 100% protection against incident HPV-16 persistent infection and against HPV-16-related cervical pathology. In 2006, Gardasil, a quadrivalent vaccine (HPV-6, -11, -16, and -18) developed by Merck was licensed for use in young women in the United States. A bivalent vaccine (HPV-16 and -18), Cervarix, developed by GlaxoSmithKline, is expected to be licensed in the United States by 2009 or 2010. Data from clinical trials consistently show that both vaccines are effective in preventing infections and lesions caused by the targeted HPV types (Koutsky and Harper, 2006).

There is widespread optimism that these vaccines will markedly reduce the burden of cervical cancer and of other HPV-related cancers at genital and nongenital sites. Research on second-generation HPV vaccines is focused on combined prophylactic and therapeutic vaccines, vaccines which could be administered orally, and vaccines which may be broadly cross-protective (Schiller and Nardelli-Haefliger, 2006).

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Human Polyomaviruses

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Polyomaviruses are small, nonenveloped DNA viruses which are widespread in nature. Although previously grouped with papillomaviruses in a single family, the International Committee on Taxonomy of Viruses has now recognized polyomaviruses as an independent virus family, the Polyomaviridae. Polyomaviruses have been isolated from many species, including humans. They are highly adapted to grow in the species they infect and have probably coevolved with their host. The first human polyomaviruses were isolated in 1971 from immunocompromised patients. JC virus (JCV) was isolated from the brain of a patient (with the initials J.C.) with Hodgkin's lymphoma who died of progressive multifocal leukoencephalopathy (PML), a demyelinating disorder of the central nervous system (Padgett, et al., 1971). BK virus (BKV) was isolated from the urine of a Sudanese renal transplant patient (with the initials B.K.) who developed ureteral stenosis (Gardner et al., 1971). Subsequent studies have shown that following clinically inapparent primary infection in childhood, both JCV and BKV persist in the kidney and in B lymphocytes. The viruses are reactivated in a variety of conditions that impair cell-mediated immune responses. Almost all of the pathologic effects of BKV and JCV infections occur in immunodeficient individuals. Very recently, two new human polyomaviruses, KI virus and WU virus, were independently detected by molecular methods in respiratory tract secretions (Allander et al., 2007; Gaynor et al., 2007). The two viruses share ~65 to 70% amino acid identity with each other but only ~15 to 50% identity with JCV and BKV. The pathogenicity and prevalence of these viruses has not yet been established. In 1960, the monkey polyomavirus, simian virus 40 (SV40), was identified in rhesus macaques. In the late 1950s and early 1960s, millions of people were inadvertently exposed to SV40 due to administration of SV40-contaminated Salk polio vaccines (Shah and Nathanson, 1976). Shortly after its discovery, SV40 was found to induce tumors in animals and to transform a variety of cell types from different species. The virus has periodically been associated with several human tumors.

CHARACTERISTICS OF THE VIRUS

Physical Properties and Genomic Organization

Polyomavirus particles measure 44 nm in diameter and have a buoyant density in CsCl of 1.34 g/cm^3. The virion is a nonenveloped, icosahedral capsid composed of three virus-encoded proteins, VP1, VP2, and VP3. The major capsid protein, VP1, accounts for more than 70% of the virion mass and has a molecular mass of 39 to 44 kDa. The capsid encloses a single molecule of circular double-stranded DNA that is in complex with cellular histone proteins. The viral DNA genome is ~5,000 bp and is organized into three functional regions, the early and late coding regions and a regulatory region (Fig. 1). The early region encodes two viral regulatory proteins, the large T and small t antigens (T-Ag and t-Ag, respectively). T-Ag possesses multiple enzymatic activities and has the ability to bind DNA and a number of cellular proteins. T-Ag regulates production of early mRNA, initiates viral DNA replication, and activates late gene transcription. Among the cellular proteins that interact with T-Ag are two proteins important for regulation of cell growth, the tumor suppressor proteins retinoblastoma susceptibility protein and p53. The interaction of T-Ag with these two proteins plays a central role in the ability of polyomaviruses to transform and immortalize cells in vitro and to induce tumors in animals. The role of t-Ag in the viral life cycle is not fully defined. The t-Ag protein is dispensable for the lytic cycle of polyomaviruses in cultured cells. It is believed that t-Ag serves an ancillary role for T-Ag activity and cell transformation. The late coding region contains the genetic information for the major structural protein, VP1, and the two minor structural proteins, VP2 and VP3. The sequence of VP3 is contained entirely with that of VP2. Based on the crystal structure of SV40 (Liddington et al., 1991), the human polyomavirus capsids are predicted to contain 360 molecules of VP1 arranged in 72 pentameric subunits. Each pentamer associates with a single VP2 or VP3 molecule to form the individual capsomeres. The late regions of BKV and JCV, but not of KI and WU viruses, encodes the agnoprotein. The SV40 agnoprotein interacts with VP1 and facilitates virion assembly and maturation (Carswell and Alwine, 1986). In contrast, the JCV agnoprotein appears to play a role in viral DNA replication and transcription and perhaps also in the dysregulation of cell cycle control and DNA repair (Safak et al., 2001). The viral noncoding regulatory region (NCRR) spans 300 to 500 bp and is located between the early and late coding regions. The NCRR contains the DNA replication origin, the TATA box, T-Ag binding sites, cellular transcription factor binding sites, and the promoters and enhancers for transcription of early and late genes.

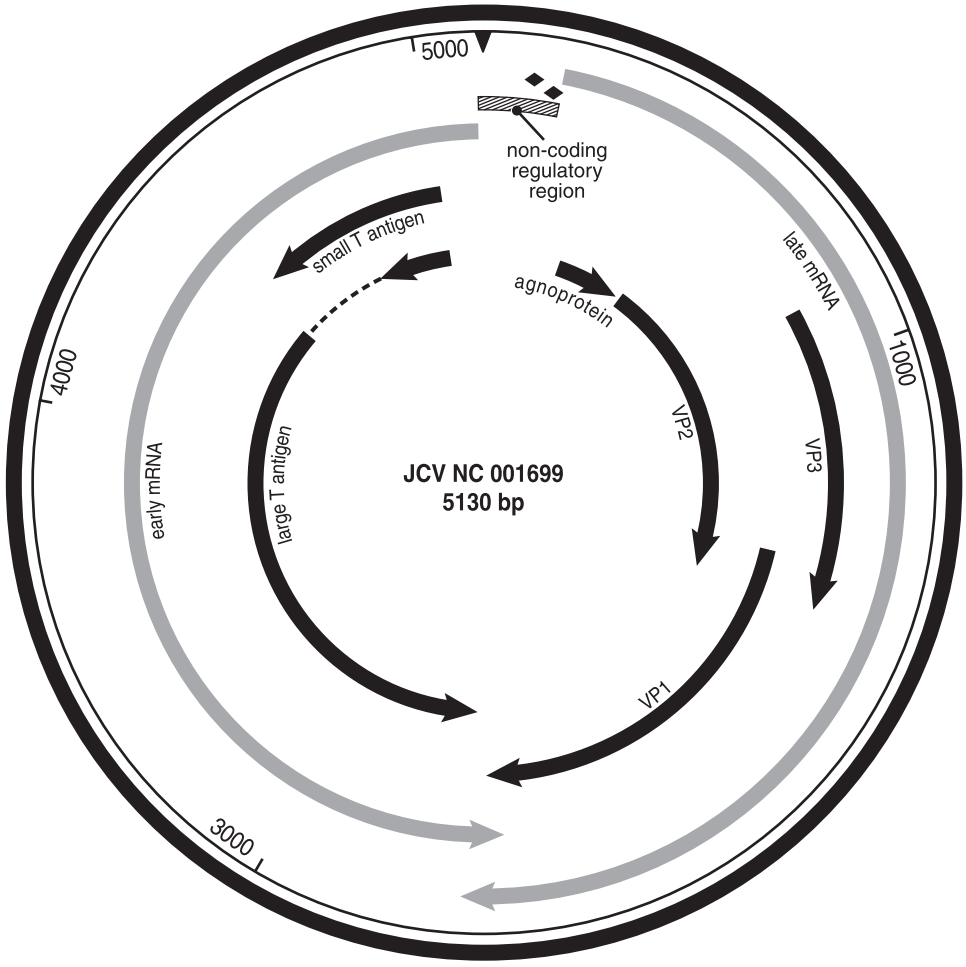

FIGURE 1 Schematic of the JCV genome. The polyomavirus genome is a closed, circular double-stranded DNA molecule approximately 5 kb in size. The early mRNA is transcribed in a counterclockwise direction and encodes the spliced T-Ag and the unspliced t-Ag genes. The late mRNA is transcribed in a clockwise direction and encodes the late genes, agnoprotein (Ag), VP1, VP2, and VP3. The genome is in black, the mRNAs are in gray arrows, and the genes are in black arrows. The noncoding regulatory region is located between the start of the early and late mRNAs, and the locations of the tandem 98-bp repeats are indicated by black diamonds.

The regulatory region contains repeat elements which are subject to deletions, duplications, and rearrangements. In general, there is a great deal of sequence variability in the NCRR among BKV and JCV variants, which is believed to confer selective growth advantages to these viruses in their hosts (Loeber and Dorries, 1988; Moens et al., 1995).

Infectious Life Cycle of Polyomaviruses

The life cycle of polyomaviruses is initiated by adsorption of virions to the cell surface. This process requires interaction with cell surface sialic acids. For BKV, an N-linked glycoprotein with α(2,3)-linked sialic acid serves as the receptor (Dugan et al., 2005), and for JCV, an N-linked glycoprotein containing terminal α(2,6)-linked sialic acid is used (Liu et al., 1998). The proteinaceous component of the cellular receptor for BKV is unknown; however, the serotonin receptor 5HT2A was recently identified as the receptor for JCV (Elphick et al., 2004). Polyomaviruses enter the cytoplasm by endocytosis. BKV enters by caveolae-mediated endocytosis (Eash et al., 2004), and JCV enters by clathrin-dependent endocytosis (Pho et al., 2000). Polyomaviruses are next transported through the cytosol to the nucleus via the cytoskeletal transport machinery. Within the nucleus, virions are uncoated and the viral minichromosome is transcribed, replicated, and subsequently encapsidated into new progeny virions. The restriction of viral tropism at the cellular level is believed to be governed in part at the transcriptional level. The transcription factors NF-κB, Tst-1, and NF-1 have been demonstrated to contribute to the neurotropism of JCV (Feigenbaum et al., 1987; Ravichandran et al., 2006). Interestingly, the NCRR of BKV contains an estrogen response element, which mediates an increase in viral promoter activity upon hormone stimulation (Moens et al., 1994). Thus, the elevated estrogen levels during pregnancy might contribute to viral reactivation and explain the increase in BKV shedding observed in pregnant women. Following viral transcription, there is a switch to DNA replication. While early transcription is believed to be a determinant of viral cell tropism, DNA replication is a determinant of species specificity. Polyomavirus T-Ag interacts with host cell DNA

polymerases in a species-specific manner (Murakami et al., 1986). Once the viral genome has been replicated, T-Ag mediates repression of early gene transcription and stimulates transcription of late genes. Expression and subsequent nuclear localization of the viral structural proteins VP1, VP2, and VP3 leads to assembly of virion capsids. Assembly occurs predominantly in the nucleus. Newly packaged virions are thought to be released by either lytic rupture of the host cell or secretion from the plasma membrane.

PATHOGENESIS AND DISEASE POTENTIAL

Primary infections with JCV and BKV occur in childhood. Infection with JCV is acquired at a later age than BKV infection. Seroepidemiological studies using highly sensitive enzyme-linked immunosorbent assays formulated with virus-like particles containing VP1 protein have shown that BKV seropositivity increases rapidly with age of children and reaches 98% at 7 to 9 years of age (Fig. 2) (Stolt et al., 2003). JCV seropositivity increases more slowly with increasing age, reaching 51% among children 9 to 11 years of age. Both viruses are endemic to almost all population areas. For reasons that are not understood, they circulate independently at both the individual and population level (Brown et al., 1975). Thus, a negative association has been noted for the presence and titer of antibody to BKV and JCV (Knowles et al., 2003). Infection, in healthy children, is most often subclinical. Serologic studies suggest that primary BKV infection may be associated with mild upper respiratory disease, but BKV has not been isolated from respiratory secretions (Goudsmit. et al., 1982). Tonsillar tissue may be the site of primary JCV infection (Monaco et al., 1998).

The viruses persist in the kidney and B lymphocytes following primary infection. It is likely that after multiplication at the site of entry, the viruses reach the kidney by a process of viremia. Infection is lifelong, as evidenced by detection of viral genomes in cadaver kidney tissues. Persistent polyomavirus infections may be associated with asymptomatic shedding of virus in the urine. Using highly sensitive PCR assays, BKV DNA can be detected in the urine of 1% of healthy persons, while JCV DNA is found in ~35% of healthy individuals, with a higher incidence of urinary JCV shedding in older individuals (Chang et al., 2002; Shah et al., 1997; Tsai et al., 1997).

Nearly all significant illnesses, as described below, due to BKV and JCV occur in immunocompromised hosts, mainly as a result of reactivation of viruses latent in the kidney. Conditions in which viruses are reactivated include pregnancy, diabetes, organ transplantation, antitumor therapy, and AIDS and other immunodeficiency diseases. Unchecked virus multiplication after primary infection of immunodeficient individuals may also lead to pathologic consequences.

PML

PML is a rare, fatal, subacute demyelinating disease of the central nervous system that results from JCV infection of oligodendrocytes in the brain (Padgett et al., 1976). It occurs as a complication of a wide variety of conditions associated with T-cell deficiencies. These conditions include lymphoproliferative disorders, such as Hodgkin's disease, chronic lymphocytic leukemia, and lymphosarcoma; chronic diseases, such as sarcoidosis and tuberculosis; primary immunodeficiency diseases; prolonged immunosuppressive therapy as, for example, in renal transplant recipients and patients with rheumatoid arthritis, systemic lupus erythematosus, and myositis; and AIDS. Most cases of PML occur in middle age or later life, but the disease is being increasingly identified at younger ages, e.g., in children with primary immunodeficiency diseases, in renal transplant recipients, and in AIDS patients. Cases of PML in the older patients are most likely the result of reactivation of latent JCV. In the younger patient, it is possible that unchecked primary JCV infection may lead to PML. The AIDS pandemic has brought about a marked increase in the number of PML cases, from less than

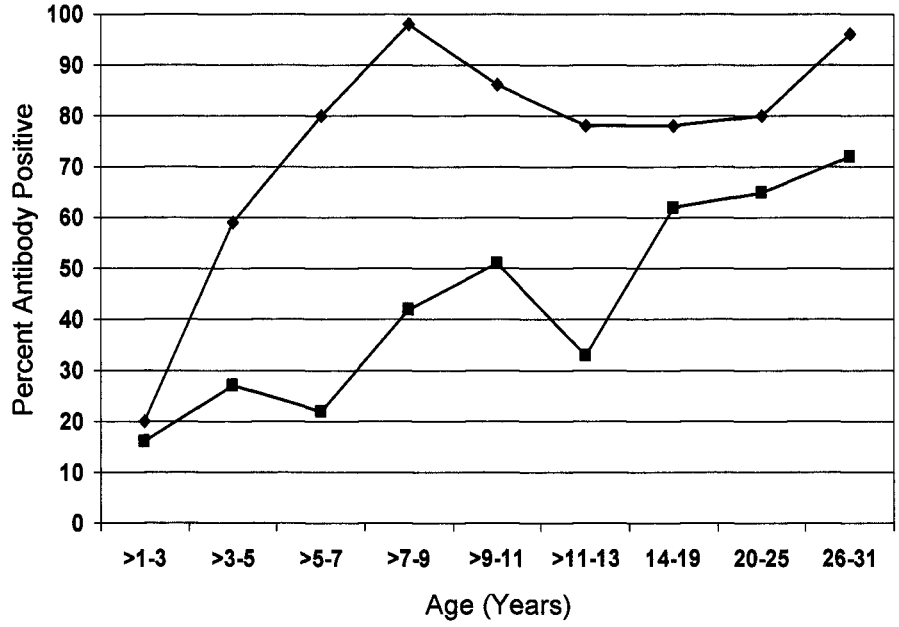

FIGURE 2 Prevalence of antibodies to BKV (◆) and JCV (■) in children (1 to 13 years of age) and pregnant women (14 to 31 years of age). (Adapted from Stolt et al., 2003.)

0.2 per million persons in 1984 to 3.3 per million persons in 1994 (Holman et al., 1998). Human immunodeficiency virus (HIV) has been estimated to be the underlying cause of immunosuppression in 55 to 85% of current cases of PML (Berger et al., 2001). PML is diagnosed in approximately 5% of AIDS cases (Berger et al., 1998). In early 2005, three patients with either multiple sclerosis or Crohn's disease developed PML in association with the administration of natalizumab, a monoclonal antibody to α4 integrin that prevents entry of inflammatory cells into brain and other tissues (Kleinschmidt-DeMasters and Tyler, 2005; Langer-Gould et al., 2005; Van Assche et al., 2005). The estimated incidence of PML was roughly 1 in 1,000 for all patients enrolled in trials of natalizumab and 1 in 300 for patients undergoing treatment with the drug for 2 or more years. Why natalizumab would uniquely predispose persons to the development of PML remains unexplained. The drug could increase the risk of PML by mobilizing latently infected B lymphocytes from the bone marrow and spleen. Alternatively, the drug may impair the cellular immune response to JCV by preventing the entry of virus-specific cytotoxic T lymphocytes into the brain or sites of viral latency.

PML has unique pathologic features resulting from cytolytic infection of oligodendrocytes with JCV (Richardson and Webster, 1983). The affected area of the brain contains foci of demyelination, which have at their edges enlarged oligodendrocytes. The nuclei of the oligodendrocytes are two to three times their normal size and basophilic, and they may contain basophilic or eosinophilic inclusion bodies. Most lesions also have bizarre, giant astrocytes with hyperchromatic pleomorphic nuclei. Inflammation is minimal or absent. Neurons are unaffected. Demyelination is a result of destruction of oligodendrocytes, which are normally responsible for the formation and maintenance of myelin sheaths. The nuclei of affected oligodendrocytes contain abundant numbers of JCV particles.

Clinically, PML has an insidious onset and may occur at any time in the course of the underlying illness. The signs and symptoms point to a multifocal involvement of the brain. Impaired speech and vision and mental deterioration are common early features of the disease. The patient remains afebrile, and headache is uncommon. As a rule, the disease is progressive, resulting in death within 3 to 6 months after onset (Fong et al., 1995). Paralysis of limbs, cortical blindness, and sensory abnormalities occur in later stages. A few patients may survive for years with stabilization of the condition and even apparent remission. A longer survival time is thought to be associated with a more marked inflammatory response in the brain. Patients who have JCV-specific $CD8^+$ cytotoxic T lymphocytes appear to survive longer than patients without such cells (Koralnik et al., 2002). The introduction of highly active antiretroviral therapy (HAART) for HIV and AIDS has had only a modest effect on the incidence of PML (Sacktor et al., 2001; Subsai et al., 2006).

The diagnosis of PML can be conclusively established by pathologic examination of a biopsy specimen or at postmortem. Macroscopically, the brain shows foci of demyelination that may vary widely in size and may become confluent and necrotic in the advanced stages of disease. The lesions are most frequent in the subcortical white matter. The cerebrum is almost always affected. Microscopically, the presence of enlarged oligodendrocyte nuclei around the foci of demyelination is diagnostic. These altered nuclei contain abundant amounts of JCV particles, antigen, and DNA. JCV particles or antigen are not found in normal brains or in nondiseased areas of PML brains. Noninvasive techniques, particularly nuclear magnetic resonance imaging (MRI) of the brain, provide an effective means for the diagnosis of PML. MRI scans of the head are nearly always abnormal in association with PML. The typical MRI abnormalities are localized to the subcortical white matter, with increased T2 signal and little contrast enhancement after gadolinium administration (Whiteman et al., 1993). Cerebrospinal fluid analysis typically shows minimal pleocytosis, less than 20 cells/μl, and only modestly elevated protein, usually amounts less than 100 mg/dl. PCR analysis of cerebral spinal fluid for JCV DNA is the best noninvasive test for confirmation of PML. Its sensitivity is ~80%, and it is highly specific (Bossolasco et al., 2005).

BKVN

BKV nephropathy (BKVN) has recently been recognized as an important cause of progressive graft dysfunction and graft loss in patients with renal allografts (Purighalla et al., 1995). It is the most common viral infection affecting renal allografts, with an incidence of ~8% and graft loss ranging from 10 to >80% (Hirsch et al., 2002). Histological features of BKVN include epithelial cells containing intranuclear inclusion bodies characteristic of BKV replication, necrosis of cells of the tubules and collecting ducts, and varying degrees of interstitial inflammation. The majority of cases occur in the first year posttransplantation. Depending on the extent of virus-induced renal injury, patients present with varying degrees of allograft dysfunction. Serum creatinine can vary from normal in the early stage of disease to a marked increase in late-stage disease with extensive renal damage. Systemic signs of infection, such as fatigue and fever, are absent. The emergence of BKVN has coincided with the expanding use of tacrolimus and mycophenolate mofetil in immunosuppressive regimens to control graft-versus-host disease. The intensity of immunosuppression appears to be a more important factor in disease than the specific agent. Host factors, such as age greater than 50, male gender, white ethnicity, and diabetes mellitus, and allograft factors, such as number of human leukocyte antigen mismatches between donor and recipient, tubular injury due to drug toxicity, proinflammatory state of the graft brought on by surgical injury, warm ischemia, and reperfusion during implantation, can also increase the risk of BKVN (Hirsch et al., 2006).

The definitive diagnosis of BKVN requires a renal biopsy showing polyomavirus-induced cytopathic changes in tubular or glomerular epithelial cells. However, because BKVN can be focal in distribution, a negative biopsy result does not rule out BKVN with certainty. A consensus conference recently proposed a histological staging scheme for BKVN (Hirsch et al., 2005). Stage A, early disease, is characterized by small numbers of tubular epithelial cells showing signs of viral replication with intranuclear inclusion bodies. In stage B, florid disease, the above findings are accompanied by signs of tubular injury and interstitial inflammation. Stage C, the late sclerosing stage, is characterized by tubular atrophy and diffuse interstitial fibrosis. Noninvasive diagnostic techniques can be used to screen for BKV replication and to make an early diagnosis of BKVN. Polyomavirus inclusion bearing "decoy cells" can easily be detected in urine with a Papanicolaou-stained cytology preparation. The positive predictive value for BKVN of the detection of decoy cells is 25 to 30%. BKV DNA can be detected in urine and plasma by PCR. Because of the high rate of excretion of BKV in urine of renal transplant recipients, the finding of BKV DNA by PCR in the urine is of limited clinical value for the diagnosis of BKVN. Testing for BKV DNA in plasma appears more

promising as a noninvasive diagnostic test for BKVN. In one recent study, PCR assays for BKV DNA in plasma had a sensitivity of 100% and a specificity of 88% for detecting biopsy-confirmed BKV nephropathy (Hirsch et al., 2002).

Hemorrhagic Cystitis in Bone Marrow Transplant Recipients

Recipients of bone marrow transplants are at high risk for hemorrhagic cystitis, with an incidence of ~30%. BKV viruria can be demonstrated in 56 to 80% of cases. An etiological role of BKV in hemorrhagic cystitis has been proposed for late-onset hemorrhagic cystitis (Arthur et al., 1986). Other factors that likely contribute to the risk of early-onset hemorrhagic cystitis include toxins, irradiation, and drugs, such as cyclophosphamide.

Primary Immunodeficiency Diseases

BKV has been isolated from the urine of patients with primary immunodeficiency diseases. A fatal end result of BKV infection has been reported. A 6-year-old boy with hyper-immunoglobulin M deficiency developed massive BKV viruria, tubulointerstitial nephritis with viral inclusions in the lesions, and irreversible renal failure (Rosen et al., 1983).

Pregnancy

BKV and JCV are reactivated in some women during normal pregnancy. In a prospective study, cytopathology in cells obtained from urine sediment suggested JCV and BKV infections in 3.2% of pregnant women (Coleman et al., 1980). This was most frequently observed in the last trimester of pregnancy. In another study, 16% of the women showed an antibody rise to one or the other virus during pregnancy (Andrews et al., 1983). All of the infections were reactivations of latent viruses in antibody-positive individuals. It has been reported that fetal sera may have BKV-specific immunoglobulin M, indicating transplacental transmission of the virus; these observations have not been confirmed.

Role in Human Malignancies

The role of polyomaviruses in human cancer is the subject of heated debate. JCV and BKV are oncogenic for laboratory animals, and they transform cultured cells (Imperiale, 2000; White and Khalili, 2004). These viruses, as well as SV40, therefore, have been investigated for their roles in human malignancies. There are reports of finding BKV, JCV, and SV40 genomes in nervous system tumors, mesotheliomas, osteosarcomas, and colon cancers. Occasionally, multifocal astrocytomas seem to arise in lesions of PML demyelination, suggesting that the tumors arose in these lesions. However, a reproducible and consistent etiologic association of JCV, BKV, or SV40 with any human malignancy has not been demonstrated.

Upper Respiratory Tract Infection

Although JCV and BKV have been detected in human tonsil tissue (Goudsmit et al., 1982; Monaco et al., 1998) and a respiratory route of transmission of polyomaviruses has been hypothesized, neither virus is considered an agent of respiratory tract disease. Recently, two new human polyomaviruses, the KI and WU viruses, were identified from nasopharyngeal aspirates of children with upper repository tract infection (Allander et al., 2007; Gaynor et al., 2007). Although genetically more closely related to each other than to any of the known polyomaviruses, the two agents are sufficiently different to suggest that they are different species. The prevalence, biology, and pathogenicity of these viruses are currently unknown. The viruses have been detected in respiratory specimens in the presence of other recognized respiratory pathogens, and thus, their role in disease is unclear. These viruses have not been detected in urine, which suggests that the biology of the KI and WU viruses in the kidney differs from that of JCV and BKV. WU virus was detected in respiratory specimens from Australia and the United States and thus appears to be geographically widespread in human populations. To date, KI virus has only been reported from Sweden.

TREATMENT

The majority of patients with BKV and JCV infections are asymptomatic and do not require treatment. There are no antiviral drugs with proven efficacy against human polyomaviruses. The mainstay of treatment for BKV nephropathy is the judicious reduction, change in drugs, or discontinuation of immunosuppressive therapy (reviewed by Hirsch et al., 2006). In general, the response to a reduced immunosuppression is better when BKVN is diagnosed early, highlighting the need for better early diagnostic markers of BKVN. A critical issue in the management of BKVN is the diagnosis of acute graft rejection because rejection is treated with steroids, which may lead to progression of BKVN. Several drugs have polyomavirus inhibitory activity in vitro, including cidofovir, leflunomide and its derivative FK-778, retinoic acids, and certain quinolone antibiotics. Although cidofovir and leflunomide have been used to treat BKVN, no controlled clinical trials have been performed to establish their efficacy. Historically, the prognosis of PML in HIV-infected patients was poor, with death occurring within 3 to 4 months of diagnosis. However, the introduction of HAART has improved survival for patients with PML (Clifford et al., 1999). To date, none of the antiviral treatments, including cidofovir, cytarabine, alpha interferon, and topotecan, that have been used in combination with HAART have shown a clearly defined beneficial effect on disease progression compared to the use of HAART alone. Paradoxically, in some patients with PML on HAART, a successful response to HAART is associated with an intense inflammatory response and a poor PML outcome.

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Herpes Simplex Viruses

LAURE AURELIAN

26

The word "herpes" is derived from the Greek "to creep." As applied to spreading, ulcerative skin manifestations, it is traced to the Hippocratic corpus, which used it to describe an assortment of cutaneous lesions including those compatible with herpes simplex virus (HSV). In 1736, Jean Astruc classified the condition as sexually transmitted and noted its high frequency among homosexuals. In the early 19th century, six clinical entities were delineated, including facial and genital herpes, but the disease was not considered communicable, probably because of the idiosyncratic appearance of symptoms in conjunction with disparate well-defined febrile illnesses. Its infectious nature was demonstrated in 1921, when lesion material passed through a filter with pores small enough to retain bacteria, was serially transmitted in rabbits. In 1929, Goodpasture concluded that a latent state is established in ganglionic neurons. We now know that two distinct viruses preferentially cause facial or genital lesions. A critical advance has been the development of antiviral chemotherapy.

CLASSIFICATION

HSV types 1 (HSV-1) and 2 (HSV-2) are members of the family *Herpesviridae*. They belong to the subfamily *Alphaherpesvirinae* and the genus *Simplexvirus*. Criteria for *Alphaherpesvirinae* classification are a variable host range, a relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and an ability to establish latency in sensory ganglia. Biological properties shared with the other members of the *Herpesviridae* are (i) common virion morphology, (ii) DNA that codes for enzymes involved in nucleic acid metabolism, (iii) DNA synthesis and capsid assembly that occur in the nucleus, (iv) production of infectious progeny that is accompanied by cell death, (v) persistence in their natural hosts, and (vi) development of immune evasion strategies that may contribute to persistence. A subset of common genes (43 core sequences) are thought to be involved in fundamental processes, including DNA replication, processing, and packaging, capsid assembly, and egress.

STRUCTURE

Capsids

Virus particles are 150 to 200 nm in diameter and consist of four components: core, capsid, tegument, and envelope (Fig. 1A). The capsid is a 100- to 110-nm protein shell with $T = 16$ icosahedral symmetry that consists of 162 capsomers (Fig. 1B). There are 3 types of capsomers: (i) hexons that form the capsid faces and edges, (ii) pentons that are located at 11 of 12 capsid vertices, and (iii) the portal for DNA entry found at one of the 12 vertices. The 150 hexons are hexamers of the major capsid protein VP5 (also known as UL19), while the 11 pentons are VP5 pentamers. The portal is a 12-mer of UL6. It is the size of a hexon (or penton), cylindrical, and has an axial channel. The portal is involved in the initiation of capsid formation. The capsomers are connected in groups of 3 by the triplexes that lie on the outer surface of the caspid floor. The capsids contain the core that contains the 152-kbp linear, double-stranded DNA that has two covalently linked components consisting of unique sequences (U_L and U_S) bracketed by inverted repeats (Fig. 1B).

Tegument

The tegument surrounds the capsid and is asymmetrically distributed. It contains at least 20 viral proteins in different stoichiometries. They include (i) virion host shutoff (vhs; UL41), which inhibits host cell translation; (ii) VP22 (UL49), which is dynamically trafficked; (iii) VP1/2 (UL36), which is associated with the release of viral DNA from incoming capsids; (iv) R1 (UL39), which is required for virus growth in non-replicating cells, including neurons; (v) immediate-early (IE) proteins ICP4 and ICP0, which have major regulatory activity; and (v) UL17, which is required for viral DNA processing and packaging. Tegument proteins enter the infected cell upon fusion of the virion envelope and cell membrane, thereby initiating the replication cascade.

Envelope

The envelope is the outer covering and is acquired from the host cellular membranes. It is decorated with spikes that contain viral glycoproteins (Fig. 1A). Eleven glycoproteins were studied (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM). HSV glycoproteins contain N-linked high-mannose and O-linked and complex heterogeneous glycans that contain sialic acid, which may be involved in entry (Teuton and Brandt, 2007).

Unique Features of the Genetic Information

The U_L and U_S components of the viral DNA can invert relative to each other. Open reading frames (ORF) are within the unique and repeat sequences that bracket them (Fig. 1B).

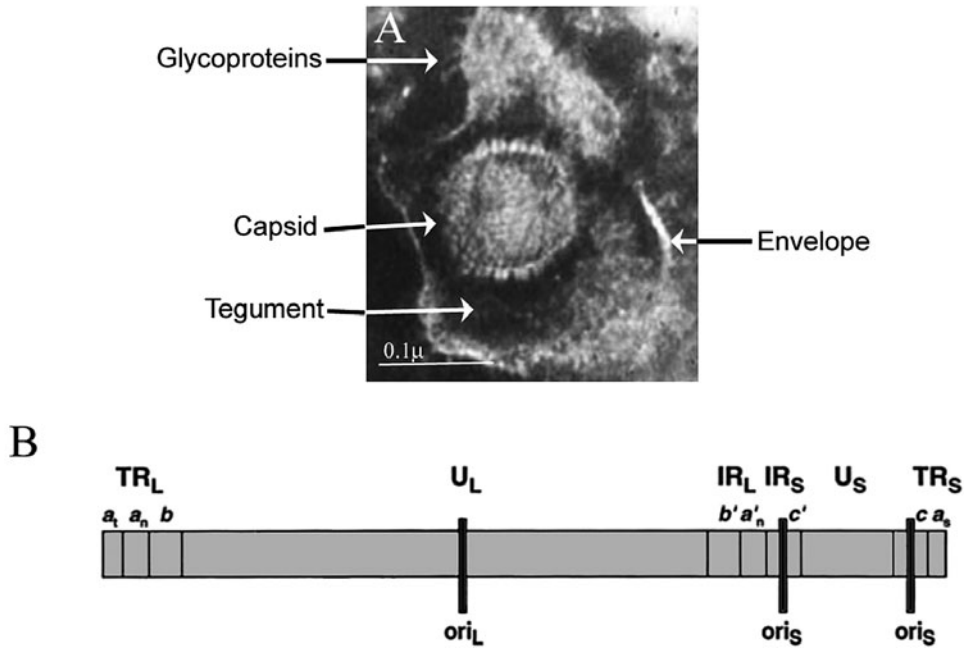

FIGURE 1 Structure. (A) Negatively stained virus particles. Capsid consists of 162 capsomers that are 9.5 by 12.5 nm (longitudinal section) and have a 4-nm-diameter channel that runs from the surface along the long axis. The capsid is surrounded by an asymmetrical fibrous-like tegument. The envelope is decorated with spikes projecting from its surface that consist of glycoproteins. Magnification, ×250,000. (B) The HSV genome has two covalently linked components consisting of unique sequences (U_L and U_S) bracketed by inverted repeats (IR_L/TR_L and IR_S/TR_S) and contains 3 origins of replication (1 ori_L and 2 ori_S).

ORFs that map in the inverted repeats are present at a rate of 2 copies/viral genome. Infected cells also contain transcripts from genome domains that are not known to specify proteins, such as the latency-associated transcript (LAT). Three sets of genes occupy both DNA strands at complementary positions, generating antisense transcripts. ORFs O and P map antisense to $\gamma_1$34.5, UL43.5 maps antisense to UL43, and UL27.5 maps antisense to gB. The expression of antisense genes is mutually exclusive (Chang et al., 1998), suggesting that they have different pathogenetic functions. Thirty-eight HSV-1 ORFs are essential for virus replication in cell culture. Less is known about HSV-2 proteins, but their function can differ relative to HSV-1. For example, in HSV-1, UL24 regulates fusogenic activity and efficient replication in neurons, potentially related to nucleolin dispersion (Lymberopoulos and Pearson, 2007). In HSV-2, UL24 is a virulence determinant (Blakeney et al., 2005). The large subunit of ribonucleotide reductase contains a functional serine/threonine protein kinase (PK) activity in HSV-2 (known as ICP10PK) that is not conserved in HSV-1. ICP10PK activates Ras and triggers survival and proliferation signaling pathways that are required for HSV-2 growth in nonreplicating cells (reviewed in Aurelian, 1998; Aurelian, 2005; Smith, 2005). Some genes (viz., $\gamma_1$34.5) contain regions with cellular homology that were, presumably, acquired during infection.

Intertypic Variation and Intratypic Polymorphism

Original DNA-DNA hybridization studies suggested that the homology of HSV-1 and HSV-2 DNA is 50%. However, based on published sequence comparisons and excluding the 2% of the alignment consisting of gaps and the divergent gG gene, which is used in serotype-specific antibody assays, the overall sequence identity in coding regions is 83% and the genomes share a similar ORF organization. Still, there remain functional differences. For example, the UL39 sequence codes for the large subunit of ribonucleotide reductase, which contains a functional PK domain in HSV-2 but not in HSV-1. Antigenic and biologic properties that differentiate between HSV-1 and HSV-2 (Table 1) suggest that the two viruses have evolved different regulatory functions. Intratypic polymorphism includes base substitutions that add or eliminate restriction endonuclease cleavage sites and/or change an amino acid. Based on the presence or absence of 225 restriction endonuclease sites, the nucleotide diversity in United States HSV-1 isolates was estimated at 0.0046 (Sakaoka et al., 1994). Restriction fragment length polymorphism variations suggest that HSV-1 variants spread to different human populations through gradual dispersion and replacement (Eda et al., 2007) and that latently infected subjects sustain exogenous reinfection (Umene et al., 2007).

REPLICATIVE CYCLE

The replicative cycle is schematically represented in Fig. 2.

Cell Entry

Four viral glycoproteins (gB, gD, gH, and gL) and the gD receptor are required for entry. Current models postulate an initial association of gC and/or gB with cell surface heparan sulfate proteoglycan, followed by the interaction of gD with one of its receptors, i.e., herpesvirus entry mediator (HVEM), a member of the tumor necrosis factor receptor superfamily, or nectin-1 or -2, which are members of the immunoglobulin superfamily. Both HVEM and nectin-1 are used by

TABLE 1 Properties of HSV serotypes

Property	HSV-1	HSV-2
Site of infection	Primarily nongenital	Primarily genital[a]
Site of recurrence	Primarily trigeminal	Primarily sacral
Transmission	Primarily nonsexual	Primarily sexual
Biochemical properties		
% G + C content of viral DNA	67	69
Overall sequence identity in coding regions	83%	
Electrophoretic mobility of viral proteins	Some differences	
Antigenic properties	Mostly cross-reactive with intratypic variation; some type-specific determinants	
Biologic properties		
Pock size CAM[b]	Small	Large
Plaque size in CE cells[b]	None or small	Yes or large
CPE in other cells	Tight adhesion of rounded cells	Loose aggregation, propensity for syncytium formation
Growth cycle		
Titers in PRK[b]	6×10^7	8×10^5
Particle-to-PFU ratio in RK cells	36	2,000
% Enveloped virus in PRK cells[c]	38	6.8
Microtubules[d]	No	Yes
Sensitivity to:		
Temp (37°C) (log loss/h)	0.07	0.27
IUdR or BUdR[e]	Sensitive	Relatively insensitive
Interferon	Relatively resistant	Sensitive
Neurotropism	More	Less
Encephalitis in immunocompetent adults	Primary cause	Rare
Virulence ($\log_{10}$ PFU/LD_{50})[f]	4.0	0.5
Latency in ganglionic neurons (% of LAT) in:		
A5	25	4
KH10	12	42[g]

[a]Significant increase in frequency of genital HSV-1 infection in last decade.
[b]CAM, chorioallantoic membrane of fertilized eggs; CE, chicken embryo cells; PRK, primary rabbit kidney cells.
[c]EM analysis reveals a high proportion of nonenveloped virus particles in HSV-1-infected cells.
[d]Formation of unique microtubule structures in HSV-2-infected cells described by EM studies.
[e]IUdR, iododeoxyuridine; BUdR, bromodeoxyuridine.
[f]Done in mice. Large variation among HSV-2 isolates. LD_{50}, 50% lethal dose.
[g]Binding to isolectin-B4 (IB4) and response to neuronal growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) have been used to define two different subgroups of nociceptive (BSL [biting, scratching, licking]) small dorsal root ganglion (DRG) neurons. A5 and KH10 neurons are two different classes of nociceptors. A5 neurons express Galβ14GlcNAc-R epitopes; KH10 neurons express Galα1-3GALβ1-4NAc-R epitopes (Margolis et al., 2007).

various clinical isolates (Krummenacher et al., 2004). In cultured cells, susceptibility to infection correlated with the number of receptors, but nectin-1 was more efficient at promoting entry than HVEM (Krummenacher et al., 2004). Entry can also be mediated by at least one defective form of nectin-1, but HSV laboratory strains that have defined gD mutations use nectin-2 for entry. Membrane fusion is required for intracellular penetration. The gD-receptor complex stabilizes the virus-cell interaction, and it acts as a trigger for fusion, which is also dependent on gB, gH, and gL. gH accounts for the fusion function. gL lacks a transmembrane domain and is believed to have chaperone-like activity necessary for the folding and transport of gH. Soluble gB binds to the cell surface independent of heparan or chondroitin sulfate proteoglycans, suggesting that entry may also be mediated by a gB-binding receptor (Bender et al., 2005). The widely expressed B5 protein was also implicated in entry, although its viral ligand is unknown (Perez-Romero and Fuller, 2005).

Incoming HSV-1 capsids are transported to the nuclear pores by cytoplasmic dynein along microtubules. Transport is facilitated by binding of the capsid protein VP26 to dynein, but microtubules and dynein were also used by a VP26-deleted mutant (Dohner et al., 2006). Entering capsids dock to the nuclear pore complex and release their DNA as a single double helix. Release is presumed to be at the portal, and it correlates with cleavage of a small proportion of the UL6 portal protein. It is unclear whether capsids differ in their ability to deliver DNA to the nucleoplasm (Newcomb et al., 2007). Neither VP26 nor 10 other tegument proteins that are essential for virus growth were required for retrograde axonal transport following infection of primary sensory neurons (Antinone et al., 2006).

Transcription and its Regulation

In the nucleus, the HSV-1 genome associates with promyelocytic leukemia protein nuclear bodies (or ND-10), giving rise to virus replication compartments. The HSV-1 genome encodes 84 proteins, 38 of which are essential for virus replication in cell culture. HSV transcripts can have common initiation signals but different termination sites, different initiation sites but coterminal ends, or different initiation

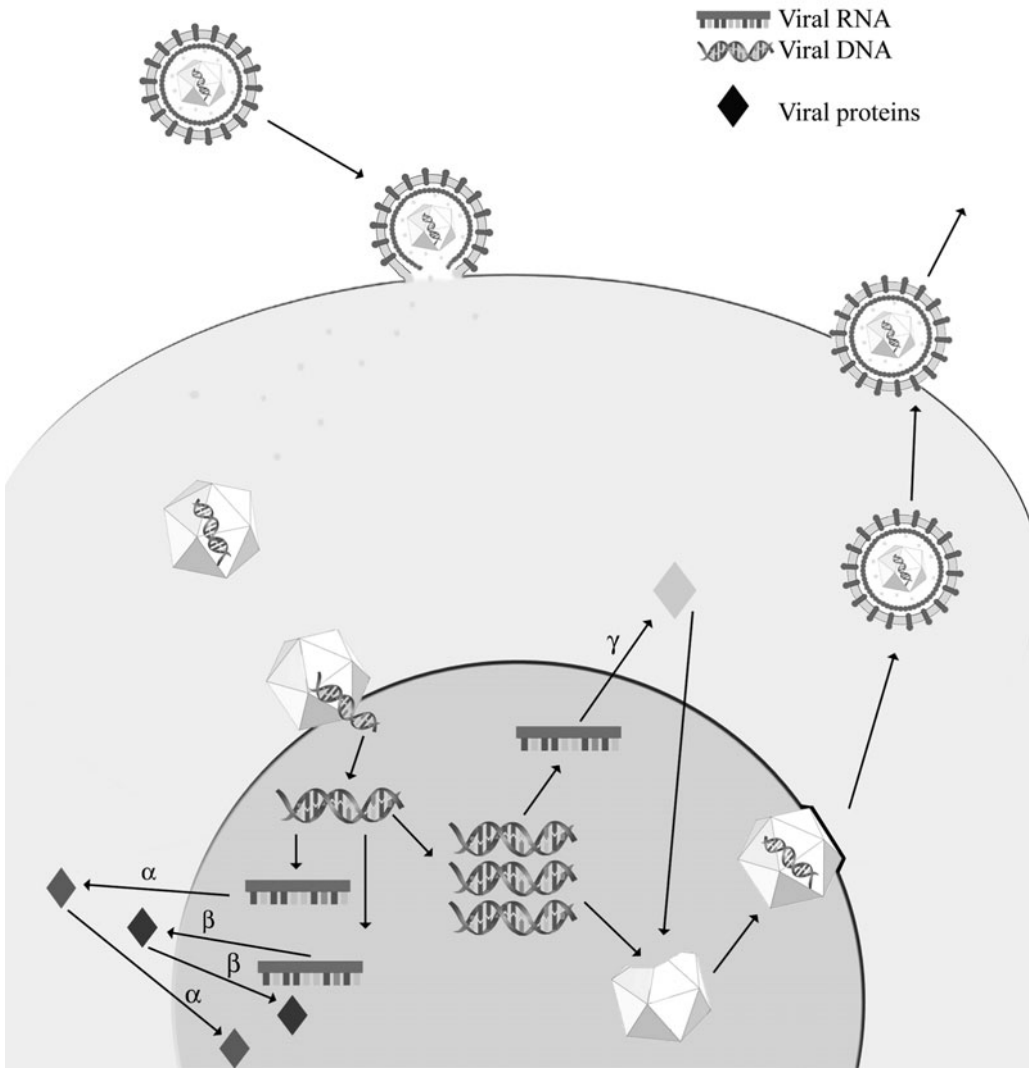

FIGURE 2 Replicative cycle. Schematic representation includes temporally regulated transcription of IE (α), E (β), and L (γ) genes and viral DNA replication.

and termination sites but partially collinear sequences. Only a few HSV mRNAs are spliced (viz. ICP0).

Three classes of genes are coordinately transcribed and temporally regulated during lytic infection: IE, early (E), and late (L) (also known as α, β, and γ, respectively). The IE proteins reach maximal levels at 2 to 4 h postinfection (p.i.), but they continue to accumulate at nonuniform rates until late in infection. IE genes are defined by (i) the presence of the sequence TAATGARAT in one or several copies within several hundred base pairs upstream of the cap site, (ii) expression in the absence of other viral protein synthesis, and (iii) enhancement of expression by the major transactivator protein VP16 that is located in the virion tegument. IE proteins ICP4, ICP27, ICP0, and ICP22 play crucial roles in the regulation of productive infection. ICP4 is required for transcription of the E and L genes (Zabierowski and DeLuca, 2004). It binds DNA, and its affinity for relatively weak binding sites is increased by DNA-dependent oligomerization aiding in transcription activation (Kuddus and DeLuca, 2007). Early in infection, ICP27 colocalizes with ICP4, and it has transcriptional and posttranscriptional activity. Late in infection, ICP27 contributes to processing and export of viral mRNA. ICP27 inhibits expression of most cellular genes, at least in part, by preventing the removal of introns from primary RNA transcripts (Smith et al., 2005). ICP0 is not essential for virus growth, but it is a major transactivator of HSV gene expression and stimulates virus growth in cells infected at a low multiplicity of infection (Everett et al., 1999). ICP0 also blocks the interferon (IFN) signaling pathway by degrading promyelocytic leukemia and dispersing the ND10 nuclear structures (reviewed in Hagglund and Roizman, 2004), and it is an HSV-1 apoptosis-triggering gene (Sanfilippo and Blaho, 2006). Over 20 years ago, we showed that ICP22 inhibition with specific antisense oligonucleotides inhibits HSV-1 growth (Smith et al., 1986). Today, it is well established that ICP22 and its in-frame carboxyl-terminal variant Us1.5 are required for virus replication in most cell types. ICP22 also affects disease pathogenesis, at least in mice (Orlando et al., 2006). Viruses lacking ICP4, ICP27, and ICP22 are growth defective

(Wu et al., 1996). The other IE protein, ICP47, shields HSV from cytotoxic T lymphocyte (CTL) activity through competitive inhibition of peptide binding to the transporter associated with antigen presentation (reviewed in Wiertz et al., 2007).

HSV genes are transcribed in the nucleus by the host cell RNA polymerase II (RNAP II). Initially RNAP II utilizes the IE promoters with the help of VP16. VP16 forms a multiprotein DNA complex with cellular proteins oct-1 and HCF to mediate transcription. It first associates with HCF, a ubiquitous transcriptional coactivator that is required for progression through the G_1 phase of the cell cycle, followed by association with oct-1 on the TAATGARAT core enhancer motif (Lai and Herr, 1997). The presence of VP16 is associated with the recruitment of transcription factors (viz., TFIIB and TFIIH) and the TATA-binding protein and assembly of the RNAP II preinitiation complex. oct-1 is critical for virus replication at low multiplicity of infection. ICP4 interacts with basal transcription factors and helps form the RNAP II transcription initiation complexes on the E and L promoters. The molecular functions of ICP27, ICP22, and ICP0 are less well defined, but ICP27 also physically interacts with RNAP II. When transcribing E and L genes, the activity of RNAP II on cellular genes is dramatically reduced, apparently involving ICP22-dependent loss of RNAP II forms that bear serine 2 phosphorylation during the IE phase of infection and a late-gene-dependent pathway during the late phase of infection (Fraser and Rice, 2007).

The E genes encode enzymes which are required for viral DNA replication, including thymidine kinase (TK) and DNA polymerase (Pol). Their synthesis reaches peak rates at 5 to 7 h p.i., and it correlates with the onset of viral DNA replication. Functional IE genes, notably ICP4, are required for E gene transcription. Controversy originally arose about ribonucleotide reductase, which consists of two subunits the expression of which is regulated with different kinetics. Because the small subunit (R2) is regulated with E kinetics and both subunits are required for enzymatic activity, ribonucleotide reductase was classified as an E gene. However, the large subunit (R1) fulfills all the criteria for IE gene classification. Its promoter has the TAATGARAT sequence characteristic of IE promoters, and its expression is regulated by the oct-1–VP16 complex independent of IE genes ICP4, ICP27, and ICP22 (Wymer et al., 1989; Desai et al., 1993). In HSV-2, IE regulation is necessary for the independently functioning PK activity, which is required for optimal expression of the IE genes ICP4 and ICP27 (Smith et al., 1998). The L genes form a continuum, differing in their kinetics and dependence on viral DNA synthesis. For example, gB and gD are synthesized relatively early in infection and are minimally affected by inhibitors of viral DNA synthesis, while glycoprotein gC is made late in infection and its expression is inhibited by inhibitors of viral DNA synthesis.

Most viral proteins are posttranslationally modified, including cleavage, phosphorylation, sulfation, glycosylation, myristylation, ADP-ribosylation, and nucleotidylation. However, with the exception of some glycoproteins (viz., gD), the extent to which processing is required for protein function and virus growth is still unclear. HSV-1 specifies at least two PKs that play significant roles in virus infection. U_S3 is involved in capsid envelopment. A smaller transcriptional unit ($U_S3.5$) that encodes a shorter, carboxyl-terminal, colinear PK is also involved, albeit with lower efficiency. U_S3 also blocks apoptosis induced by defective viruses or exogenous agents, while $U_S3.5$ does not (Poon et al., 2006). HSV-1 with a deletion of U_S3 is defective in the ability to prevent lysis of target fibroblasts, suggesting that U_S3 is involved in this aspect of immune evasion (Aubert and Krantz, 2006). The other PK involved in the HSV-1 lifecycle, UL13, regulates UL34 and UL31 localization, either by phosphorylating U_S3 or by a U_S3-independent mechanism (Kato et al., 2006). UL13 also phosphorylates the IE protein ICP22 and the viral Fc receptor and complexes with the glycoprotein gE (Purves et al., 1993; Ng et al., 1998). The contribution of these PKs to HSV-2 pathogenesis is not equally well understood. However, HSV-2 also specifies another PK (ICP10PK) that phosphorylates the R2 subunit of ribonucleotide reductase and the cellular proteins RasGAP. The latter contribute to the ability of ICP10PK to activate the Ras signaling pathway which is required for HSV-2 growth (Smith et al., 1992; Smith et al., 1998; Smith et al., 2000).

Virus DNA Replication

HSV DNA replication is first detected at 3 h p.i., but the bulk occurs at 5 to 8 h p.i. Replication initiates in an origin-dependent manner and continues perhaps by a rolling circle and/or homologous recombination-driven mechanism. Homologous recombination may help maintain the integrity of the viral genome through repair of mutated or damaged DNA and is likely to contribute to the intratypic molecular diversity of the viral genome. The majority of the replicative intermediates are long concatemers apparently generated through sequence replacement or insertion. They contain genomic units with U_L and U_S segments in different orientations that give rise to four possible isomers in equal molar ratios. Isomerization is believed to result from recombination triggered by breakage at the *a* sequence in the inverted repeats, a recombinational hot spot. Endonuclease G appears to be the only cellular enzyme that can specifically cleave the *a* sequence, and it has been proposed that it initiates the *a* sequence-mediated inversion of the U_L and U_S components during HSV-1 DNA replication (Huang et al., 2002).

Seven viral proteins are essential for the replication of the HSV-1 genome: the origin-binding protein (UL9) that is only required early postinfection, the single-stranded DNA-binding protein (UL29, also known as ICP8), the helicase-primase heterotrimer (UL5, UL8, and UL52), and Pol (UL30) and its processivity subunit (UL42) (reviewed in Wilkinson and Weller, 2003). The HSV genome contains 3 origins of replication with high-affinity binding sites for UL9: a single copy of Ori_L located in the middle of the U_L segment, and two copies of Ori_S located in the repeats flanking the U_S segment (Fig. 1B). Mutants lacking Ori_L or both copies of Ori_S are replication competent, indicating that Ori_L and Ori_S can compensate for each other, but they may differ in their functional efficiency. Ori_L may be involved in HSV-1 morbidity, mortality, and latency reactivation in mice (Balliet and Schaffer, 2006). UL42 possesses both Pol- and DNA-binding activities, with the latter required for DNA synthesis. UL42 also exerts substantial effects on the fidelity of DNA replication (Jiang et al., 2007). The DNA replication compartments contain cellular proteins that participate in DNA metabolism. One of these is the hyperphosphorylated replication protein A, which plays a key role in the recognition, signaling, and repair of damaged DNA. Pol is involved in inhibition of replication protein A hyperphosphorylation, thereby avoiding activation of the host stress response that may be detrimental to viral genome

replication (Wilkinson and Weller, 2005). The Hsp90 chaperone system prevents Pol degradation and its aberrant folding and/or mislocalization to the cytoplasmic compartment (Burch and Weller, 2005).

Virion Assembly

Assembly occurs in the nucleus (Fig. 3A). Three types of capsids (A, B, and C) can be isolated from the nuclei of infected cells and are detected by sucrose gradient centrifugation. C capsids contain the viral DNA and sediment furthest down the gradient. B capsids contain the internal scaffold proteins, and A capsids are empty. All three have mature shells, but A and B seem to be aberrant by-products of assembly. The capsid is first assembled as a spherical precursor particle known as procapsid into which DNA is then packaged. The surface shell of the procapsid is composed of hexamers and pentamers of VP5 coordinated by triplexes of UL18 (VP23) and UL38 (VP19C) with the UL6 portal at one vertex. The viral genome is inserted by a molecular motor made up of the portal vertex protein UL6 together

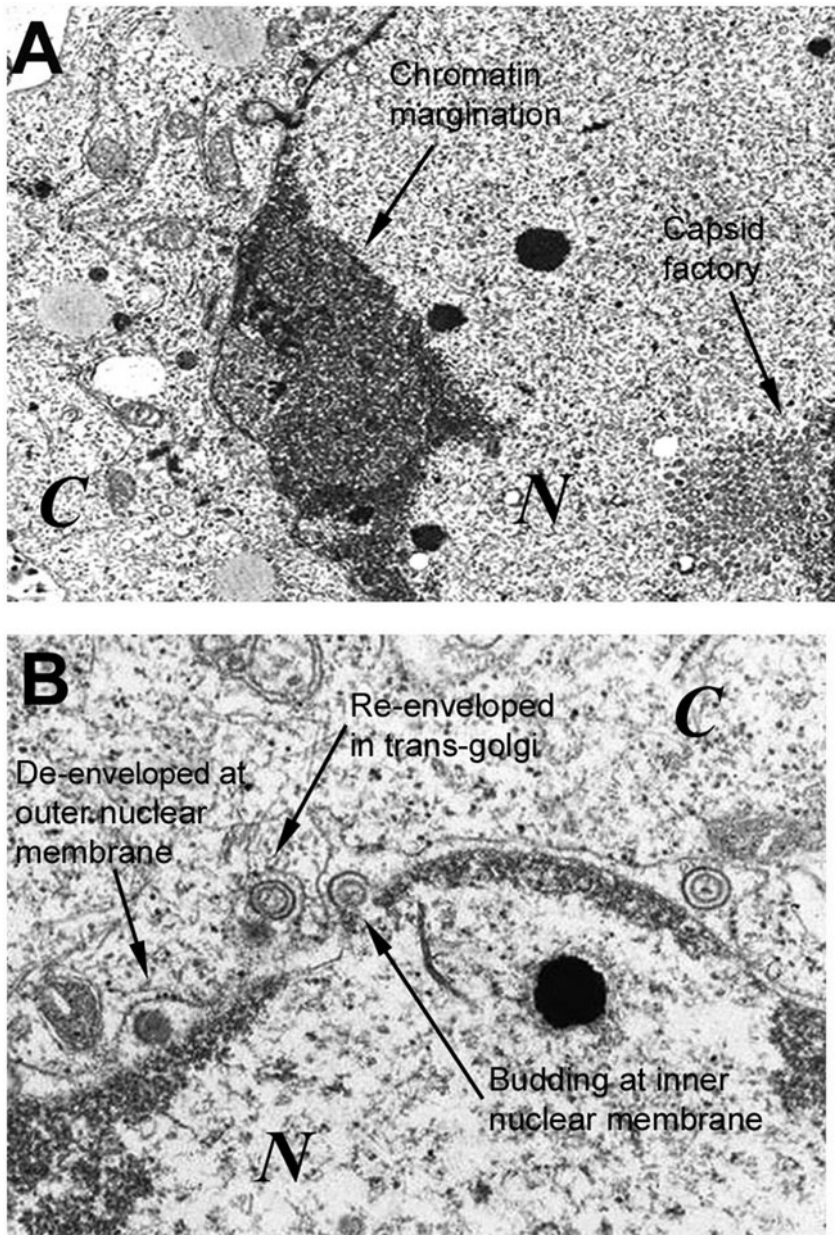

FIGURE 3 Virus replication. Thin sections of HSV-2-infected cell (12 h after infection) stained with uranyl acetate and lead citrate show nuclear (*N*) and cytoplasmic (C) compartments. (A) Intranuclear capsids are in different stages of assembly, and the nucleus shows chromatin margination. (B) Virion assembly documents budding through the inner nuclear membrane, de-envelopment at the outer nuclear membrane, and reenvelopment in the *trans*-Golgi network. Magnification, ×55,700.

with terminase (consists of UL15, UL28, and UL33), which recognizes packaging signals on DNA, cleaves replicated concatemeric DNA into monomeric units, and translocates the genome units into the capsid (Jacobson et al., 2006). UL15, which is highly conserved in the herpesviruses, shares similarity to bacteriophage terminases and probably functions as the catalytic subunit of the DNA terminase complex. UL15 interacts with UL6 and UL28, both of which are only transiently associated with capsids and are missing from the DNA-containing capsids. The function of UL33 in the cleavage-packaging reaction is still unclear. During packaging, the protein scaffold within the procapsid is cleaved and removed, and the procapsid switches its shape from spherical into a more robust and angular polyhedral structure as the hexamers are remodeled and sites around their outer tips are created for binding of VP26 (UL35). The interaction between VP5 and the scaffold proteins is important for assembly of an icosahedral capsid structure (Huang et al., 2007). Protein UL17 is required for cleavage of the replicating DNA concatemer to genome-sized pieces and DNA packaging, while UL25 stabilizes capsid-DNA structures (Trus et al., 2007).

Envelopment and Cell Egress

The mechanism of egress of the alphaherpesviruses has been the subject of long-standing controversy. According to one model, DNA-containing capsids undergo envelopment by budding through the inner nuclear membrane and are transported within vesicles from the outer nuclear membrane through the endoplasmic reticulum and secretory Golgi pathway to the cell surface. Because this model does not account for the lack of identity between primary and mature virions, the second model proposes that primary envelopment (at the inner nuclear membrane) is followed by de-envelopment of the capsids at the outer nuclear membrane and secondary reenvelopment in the *trans*-Golgi network. Here the capsids acquire their mature envelope by budding into a cytoplasmic organelle that transports them to the plasma membrane. Release is by exocytosis (Fig. 3B).

Some aspects of primary envelopment have been elucidated. The viral protein complex UL31/UL34 recruits PKC to the nuclear membrane, and it phosphorylates lamin B, resulting in lamina permeabilization and providing sites on the inner nuclear membrane where HSV capsids become enveloped. U_S3 is involved in primary envelopment. It localizes to the nuclear envelope and largely determines the phosphorylation state and localization of the necessary primary envelopment factor, the UL34 protein. However, the significance of UL34 phosphorylation is cell type dependent, and efficient viral morphogenesis also requires U_S3-mediated phosphorylation of an infected cell protein other than UL34 (Ryckman and Roller, 2004; Poon et al., 2006). Phosphorylation of the inner nuclear membrane protein emerin, by an as yet unknown kinase, reduces its association with the membrane, contributing to nuclear egress (Morris et al., 2007). Glycoproteins gB, gD, gC, and gM present in the nuclear membrane interact with the tegument-coated capsids and become incorporated into the virion envelope upon budding through the inner nuclear membrane (Baines et al., 2007). Either gB or gH can promote the fusion between the virion envelope and the outer nuclear membrane, facilitating budding. This is in direct contrast to HSV entry, which requires both gH and gB, suggesting that different types of fusion are required for entry and egress. The secondary envelopment requires glycoproteins gE/gI, and gD, acting in a redundant fashion (Farnsworth et al., 2007). UL20 and glycoprotein gK contribute to secondary envelopment, and their interaction is necessary for intracellular protein trafficking, virus egress, and virus-mediated cell-to-cell spread (Foster et al., 2004). Vps4, an enzyme that is essential for the formation of luminal vesicles in multivesicular endosomes, is also required for the cytoplasmic envelopment of HSV-1 (Crump et al., 2007). During latency reactivation, HSV-1 envelopment likely occurs in neuronal varicosities and growth cones, where the primary site is the *trans*-Golgi network and traffic and egress are along microtubules. In epithelial cells and neurons, newly assembled virions are directed to specialized cell surface domains (viz., epithelial cell junctions and neuronal synapses) for rapid entry into adjacent connected cells (cell-to-cell spread). Glycoproteins gE/gI bind ligands or receptors at these sites, promoting spread.

Nuclear and cytoplasmic sites have been proposed for the incorporation of different tegument proteins. Using immunogold electron microscopy, VP16 was seen in primary enveloped virions that had not yet initiated de-envelopment and reenvelopment, while VP16, VP22, and VP13/14 were seen in the mature extracellular virions, implying that VP16 may be acquired both during primary and secondary envelopment (Naldinho-Souto et al., 2006). This is also true for UL17 (Thurlow et al., 2005). Infected cells contain two forms of vhs, which differ in the extent of phosphorylation (58 and 59.5 kDa), but only the 58-kDa form is present in enveloped cytoplasmic virions. It appears that the processed 59.5-kDa product is transported to the nucleus, binds intranuclear capsids, and is converted to the 58-kDa form at some stage before final envelopment. Alternatively, the 58-kDa form is packaged through interactions with other tegument proteins in the cytoplasm or viral envelope proteins at the site of final envelopment (Read and Patterson, 2007).

Host Cell Effects

The IE proteins inhibit cellular DNA synthesis and cause cell cycle arrest. Onset of E gene expression coincides with irreversible shutoff of host cell macromolecular syntheses, which is primarily mediated by vhs. vhs degrades cellular mRNA through an endoribonuclease activity regulated, at least in part, through complexation with VP16. Virus replication is associated with cell rounding, chromatin margination, and the formation of nuclear inclusions (Fig. 3). The earliest manifestation of host cytopathic effects (CPE) is an enlarged peripherally displaced nucleolus. Concurrently, there is chromatin margination and the nucleus becomes multilobed. A basophilic Feulgen-positive inclusion body that contains viral DNA is seen early in infection; an eosinophilic intranuclear inclusion body (type A) devoid of virus material develops late in infection. Cellular membranes are altered, giving the impression of reduplication late in infection (Fig. 3). Cell surface changes include the acquisition of a receptor for the Fc domain of immunoglobulin, a receptor for C3b, a fragment of the third component of complement, and antigenic determinants that are major targets of the immune response to the virus.

Most HSV-1 and HSV-2 strains cause cells to round up and attach to each other, but some strains cause cell fusion (polykaryocytosis). Mutations that confer this phenotype map in at least 5 loci in the HSV-1 genome, within gB, gK, gL, UL24, and UL20 (Roop et al., 1993). Polykaryocytosis is the cell alteration used in the cytologic diagnosis of HSV. The gE-gI glycoprotein complex interacts with components of cell junctions and may be involved in intercellular virus

spread (Dingwell and Johnson, 1998). Apoptosis is a sequentially ordered response to intracellular and extracellular factors that culminates in cell death. HSV-1 triggers apoptosis at multiple metabolic checkpoints, and in turn, it has evolved mechanisms to block apoptosis at each point, in a cell-type-dependent manner (reviewed in Aurelian, 2005; Nguyen and Blaho, 2007). Virus infection also induces expression of cellular proteins in a cell-type-specific fashion. For example, in microglia, which are resident monocyte-like cells in the brain, an HSV-2 mutant with a deletion of ICP10PK (ΔPK) induces proinflammatory cytokines (viz., tumor necrosis factor alpha), while a mutant with a deletion of the ribonucleotide reductase domain in ICP10 (ΔRR) induces anti-inflammatory cytokines (viz., interleukin 10 [IL-10]). This is associated with the ability of ΔRR, but not ΔPK, to protect from excitotoxin-induced brain inflammation (Laing and Aurelian, 2008). Further studies of host cell gene regulation are needed to better understand its relationship to disease causation.

EPIDEMIOLOGY AND ASSOCIATED RISKS

Primary HSV-1 infection usually occurs by 5 years of age and is generally asymptomatic. There is no seasonal variation in the incidence of infection. It is estimated that 50 million adults have oral herpes in the United States. Only 10 to 15% of primary infections produce clinical disease. Clustered outbreaks of HSV stomatitis were reported within orphanages and hospitals (Hale et al., 1953). Virus shedding in the saliva is a significant reservoir for transmission. HSV-1 can be isolated from the mouth for 7 to 10 days after primary infection. Shedding decreases with advancing age, with salivary excretion reported in 2 to 9% of asymptomatic adults. Geographic location, socioeconomic status, and age influence the frequency of HSV infection. Presumed middle class individuals in industrialized societies become infected later in life, with prevalences of 40 and 60% in the second and third decades of life, respectively. Only 5 of 18 countries surveyed in the National Health and Nutrition Examination Survey (NHANES) had an HSV-1 antibody prevalence less than 70% (Nahmias et al., 1990). At the time of NHANES III (1988 to 1994), two-thirds of the U.S. population 12 years and older had HSV-1 antibody. Prevalence increased with age and varied by race and ethnicity; the majority of persons in all racial and ethnic groups were HSV-1 seropositive by age 30. Overall, there was only a minimal decrease in the national seroprevalence of HSV-1 after 1980, from 62.0% (95% confidence interval [CI], 59.6 to 64.6%) in 1988 to 1994 to 57.7% (95% CI, 55.9 to 59.5%) in 1999 to 2004 (Schillinger et al., 2004). Among persons infected with HSV-1, but not with HSV-2, a higher percentage reported having been diagnosed with genital herpes in 1999 to 2004 than in 1988 to 1994 (1.8% versus 0.4%, respectively; $P < 0.001$), suggesting that the incidence of genital herpes caused by HSV-1 may be increasing (Xu et al., 2006). In U.S. college students, the proportion of newly diagnosed genital HSV-1 infections increased from 31% in 1993 to 78% in 2001 ($P < 0.001$). HSV-1 was more common in females than males and in persons aged 16 to 21 years than ≥22 years. Multiple logistic regression models showed that HSV-1 was associated with orogenital contact ($P < 0.001$) and a monogamous relationship in the last 2 months (Nieuwenhuis et al., 2006). A recent cross-sectional survey of the general population in eight European countries revealed intercountry and intracountry differences in age-standardized HSV-1 seroprevalence (52 to 84%), but a significant proportion of recently screened adolescents were HSV-1 seronegative.

The risk of HSV-2 infection is correlated with markers of sexual promiscuity, including early age of first intercourse, large numbers of sexual partners, history of other sexually transmitted diseases, and increasing age (Nieuwenhuis et al., 2006). The highest prevalence of HSV-2 antibodies (75 to 98%) was seen in female prostitutes and homosexual men. Women are about 45% more likely than men to be infected with HSV-2. The risk of susceptible females for contracting HSV-2 from infected males is 80% after a single contact. The probability of infection with HSV-2 is less than 10% for U.S. women having one partner. It increases to 40%, 62%, and >80% as the number of partners increases to 2 to 10, 11 to 50, and >50, respectively. For heterosexual men, these risks are 0%, 20%, 35%, and 70% for each of the risk groups, respectively. For homosexual men, the risks are >60% and 90% for those with 11 to 50 and >50 partners, respectively (reviewed in Ashley and Wald, 1999). Comparison of NHANES data indicates that the overall age-adjusted HSV-2 seroprevalence was 17.0% (95% CI, 15.8 to 18.3%) from 1999 to 2004 and 21.0% (95% CI, 19.1 to 23.1%) from 1988 to 1994, a relative decrease between the 2 surveys ($P < 0.001$). Decreases in HSV-2 seroprevalence were especially concentrated in persons aged 14 to 19 years between 1988 and 2004, suggesting that the trajectory of increasing HSV-2 seroprevalence in the United States may have been reversed (Xu et al., 2006). In multivariate analyses, circumcision did not affect the rate of HSV-2 infection (Xu et al., 2007b). However, in a population consisting primarily (>90%) of African American men, the HSV-1 and HSV-2 seropositivities were 61% and 45%, respectively, and the overall viral shedding rate was 21.1%. The rate of asymptomatic shedding was 5.2%, suggesting that genital HSV infections are still common and largely unrecognized among this segment of the population, and HSV-1 infection constitutes a nontrivial proportion of the infections (Sizemore et al., 2006). Most men do not report genital HSV disease or are unaware that they are infected (Sizemore et al., 2005).

HSV-induced encephalitis (HSE) is the most common viral encephalitis with an estimated incidence of 2.3 cases per million population per year. The age distribution is biphasic with one peak at 5 to 30 years of age and a second peak >50 years of age. HSV-1 accounts for 95% of adult cases, and the majority are due to reactivation from latency. HSV-2 does not generally cause encephalitis in adult humans, but the virus can cause aseptic meningitis, predominantly with primary infection. HSV-2 was isolated from the cerebrospinal fluid (CSF) in 0.5 to 3% of patients with aseptic meningitis.

Following our and other studies dating back to the late 1980s (Aurelian, 1990), the dialogue between HSV-2 and human immunodeficiency virus (HIV) infections is continuing. HSV-2 infection was associated with a threefold-higher rate of HIV acquisition by meta-analysis calculation of age and sexual behavior-adjusted relative risks. However, this may differ for distinct populations (Cachay et al., 2007; Kapiga et al., 2007), potentially reflecting different HIV compartmentalization and its effect on T-cell replication. While this is still unresolved, it is evident that HSV-2 and HIV can occupy the same space while pursuing distinct chronicity pathways. In cultured cells, HSV-2 and HIV coreplicate and gB/gD were colocalized with HIV gp160 without (Legoff et al., 2007) or with (Calistri et al., 1999) pseudotype formation. In addition, HSV activates HIV-1 gene expression in macrophages that harbor latent HIV

(Feng et al., 1993), potentially contributing to HIV pathogenesis at the level of immune responses.

TRANSMISSION AND TISSUE TROPISM

HSV is transmitted during close personal contact. The risk of transmission is directly related to virus load. Virus shedding in the saliva was reported in 20% of children 7 to 24 months old, 18% of those 3 to 14 years old, 2.7% in those ≥15 years old, and 2 to 9% of asymptomatic adults. HSV-1 was isolated from the mouth for 7 to 10 days after primary infection. Oral secretions from 36 to 45% of patients examined at prodrome (24 to 48 h before clinical recurrence) were HSV-1 positive by PCR. HSV-1 may also be excreted in tears from asymptomatic individuals. Genital HSV-1 results from self-inoculation or from oral sexual practices. Sexual contact is the primary route of HSV-2 transmission. Asymptomatic shedding, first reported by us (Aurelian and Kessler, 1985; Aurelian et al., 1990), is now considered the major cause of transmission. Half of the time it occurs more than 7 days before or after a symptomatic recurrence. However, most current studies of asymptomatic patients use PCR, the sensitivity of which is at least four- to fivefold higher than that of virus isolation. When virus titers were measured, they were 100 to 1,000 times higher in clinical lesions than in secretions from asymptomatic subjects, suggesting that the risk of transmission is much higher upon contact with active lesions than asymptomatic shedding. In fact, cervical lesions are generally unnoticed (Fig. 4E) and may be confused with asymptomatic shedding. In discordant

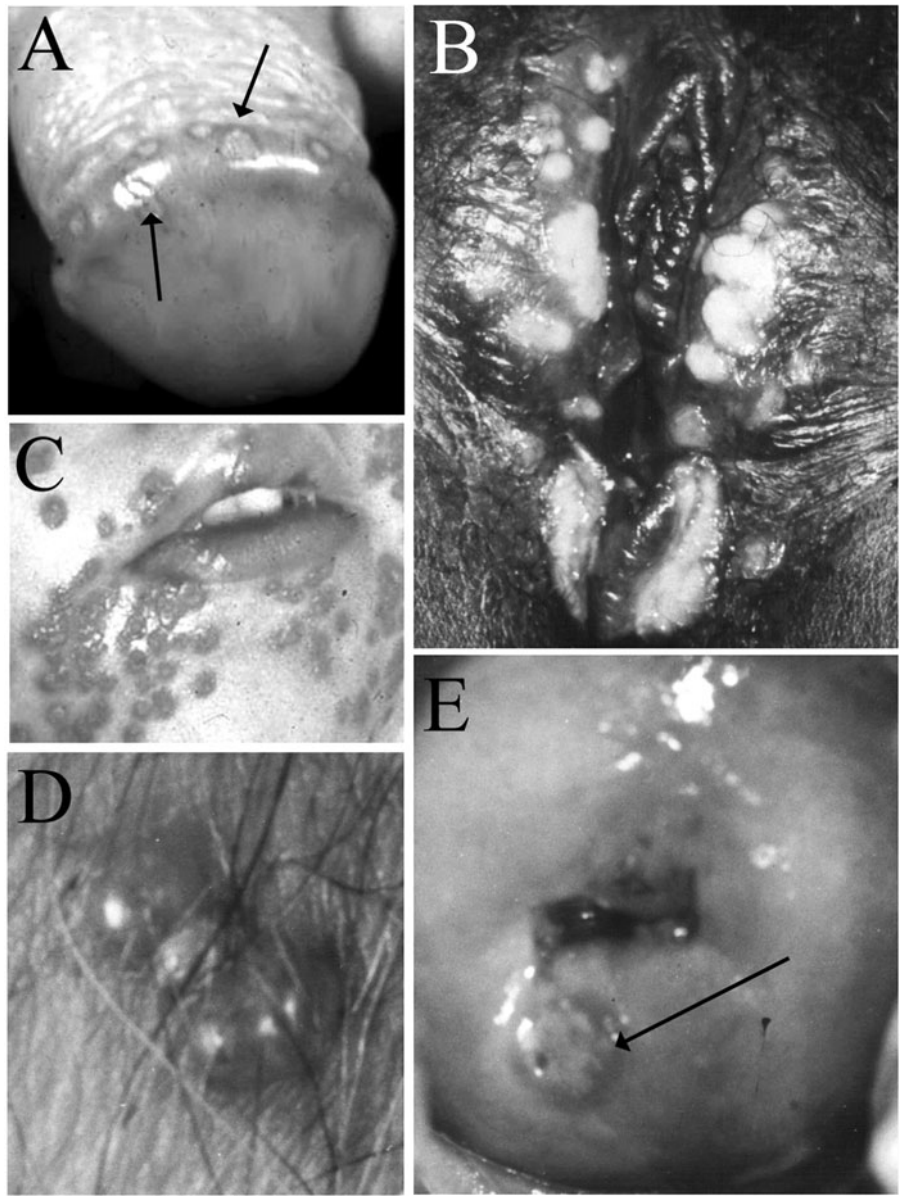

FIGURE 4 Clinical manifestations. HSV-2-induced lesions of the penis (A), vulva (B and D), and cervix (E). Clinical HSV-1 manifestation is gingivostomatitis (C).

couples, 50% of seronegative partners remained seronegative for 16 months of contact, but use of condoms or daily suppressive therapy may be an option to reduce the risk of transmission. Intrapartum transmission accounts for 75 to 80% of neonatal HSV cases.

In children and young adults, HSE usually results from primary infection by virus entering the central nervous system (CNS) through neurotropic spread by way of the olfactory bulb. The hematogenous route of infection is common in immunosuppressed patients and neonates. Murine studies suggest that gender influences hematogenous HSV-1 infection in the CNS, and apolipoprotein E is involved in HSV-1 colonization of the brain from the blood. Retrospective analysis of administrative claims from the Integrated Health Care Information Services National Managed Care Benchmark data indicated that, of 233,487 infants born to 252,474 mothers during January 1997 to June 2002, the numbers of infants assigned a code reflecting possible neonatal infection at ≤30 and ≤90 days after birth were 178 (0.08%) and 338 (0.15%), respectively. Of the 338 mothers in the latter group, 12% had a prior HSV diagnosis, 5% were prescribed antiviral therapy, and 3% used antiviral medication and had a cesarean delivery. National surveillance of neonatal HSV is required to better elucidate its incidence and improve prevention and treatment (Whitley et al., 2007). Postnatal infection as a consequence of nursing on an infected breast was also documented (Dunkle et al., 1979). During late pregnancy, HSV-2 can, in rare cases, cause severe intrauterine infections, which include brain and skin lesions, general infection, and disseminated intravascular coagulation and are often fatal (Törnhage et al., 2007).

CLINICAL FEATURES AND PATHOGENESIS

HSV-1 and HSV-2 exhibit similar clinical manifestations. They infect neonates, children, and adults and produce a wide spectrum of diseases, including mucous membrane and skin lesions and ocular, visceral, and CNS disease (Table 2). The incubation period is 1 to 26 days (median, 6 to 8 days). The severity varies among individuals, ranging from asymptomatic to fatal. Age, gender, genetic factors, immune competence, associated illnesses, and virulence of the infecting virus strain influence severity. The HSV-1 portal of entry is generally the oropharyngeal mucosa. Following replication at the site of infection, the trigeminal ganglia are invariably colonized (latency). Most (70 to 90%) childhood HSV-1 infections are asymptomatic. In children 1 to 3 years of age, a major manifestation is gingivostomatitis, a serious infection of the gums, tongue, mouth, lip, facial area, and pharynx, often accompanied by high fever, malaise, myalgias, swollen gums, irritability, inability to eat, and cervical lymphadenopathy (Fig. 4C). Later in life, the major HSV-1 clinical manifestation is an upper respiratory tract infection, generally pharyngitis, and a mononucleosis-like syndrome. Recurrent skin lesions are the hallmark of HSV pathogenesis and they follow clinically overt or asymptomatic primary infections. Nearly all people with clinically recognized primary HSV-2 and 55% of those with clinical HSV-1 infection develop at least one recurrent episode within 1 year after the primary infection. Reactivated HSV-1 is associated with mucosal ulcerations or lesions at the mucocutaneous junction of the lip, presenting as small vesicles that last 4 to 7 days and are often referred to as herpes labialis, facialis, or febrilis, cold sores, or fever blisters. With the exception of severely immunocompromised patients, recurrent herpes is relatively benign, producing fewer and smaller lesions that heal faster than those seen in the primary infection. Systemic symptoms are rare, and the duration of virus shedding is shorter. A prodrome often signals recurrence and it is characterized by a tingling sensation at the site at which the lesions will appear that may precede it by a few hours to 2 days. This sensation may be accompanied by radiating radicular pain. Virus reactivation evidences regional specificity, with HSV-1 reactivating most frequently from trigeminal ganglia and HSV-2 from sacral ganglia (Lafferty et al., 1985).

Other skin diseases generally associated with HSV-1 include primary herpes dermatitis (a generalized vesicular eruption), eczema herpeticum (a manifestation of a primary infection in which the skin is the portal of entry), and traumatic herpes (resulting from traumatic skin breaks due to burns or abrasions). Herpetic whitlow is an occupational hazard (among dentists, hospital personnel, and wrestlers [herpes gladiatorum]) resulting from infection of broken skin (often on the fingers). HSV-1- and HSV-2-associated erythema multiforme (HAEM) is a recurrent skin disease that follows reactivation of latent virus or primary infection and occurs at distal sites. HAEM lesions contain HSV DNA fragments (most often comprising Pol) that are delivered by migrating infected $CD34^+$ stem cells. Lesion development is initiated by virus gene expression, notably Pol, but is devoid of replicating virus. Symptoms are associated with virus-specific and autoimmune T-cell responses. The

TABLE 2 Spectrum of diseases associated with or caused by HSV

Stomatitis
Herpes labialis
Genital lesions
Anal, rectal lesions
Atypical and hyperproliferative lesions
Primary herpetic dermatitis
Eczema herpeticum
Traumatic herpes
Herpetic whitlow
Herpes gladiatorum
Persistent mucocutaneous lesions
Acute herpetic rhinitis
Keratoconjunctivitis
Keratitis
Chorioretinitis
Neonatal herpes
Meningitis
Mollaret's meningitis
Encephalitis
Bell's palsy
Progressive dementia syndrome
Monofocal epilepsy
Necrotizing myelitis
Pharyngitis
Hepatitis
Pneumonitis
Urethritis
Cystitis
Monoarticular arthritis
Adrenal necrosis
Atherosclerosis
Autonomic system dysfunction
Peptic ulcer
HAEM

virus-specific component consists of a restricted population of Th1 cells (primarily, $CD4^+$ Vβ2) that produce IFN-γ. The autoimmune component is in response to antigens released by lysed or apoptotic virus-infected keratinocytes. A genetic predisposition is implicated (Aurelian et al., 2003; Ono et al., 2005). Herpes folliculitis is a rare HSV-1 manifestation that often presents with lymphocytic folliculitis devoid of epithelial changes characteristic of virus infection. Acute HSV-1 rhinitis is a primary infection of the nose recognized by the appearance of tiny vesicles in the nostrils usually associated with fever and enlarged cervical lymph nodes. Both HSV-1 and HSV-2 were isolated from the posterior pharynx in 11% of pharyngitis cases. Concomitant lesions of the tongue, buccal mucosa, and gingiva were seen in one-third of cases. HSV-1 infections of the eye can lead to stromal keratitis, one of the leading causes of blindness in the western world. HSV keratitis also can be acquired after penetrating keratoplasty (incidence is 1.2 per 1,000 person years) occurring in the first 2 years after transplantation (Remeijer et al., 1997). This disease has a virus-specific T-cell component ($CD4^+$; T helper type 1 [Th1]) (Niemialtowski and Rouse, 1992). Chorioretinitis is a manifestation of disseminated HSV infection that may occur in neonates or in patients with AIDS.

Initial HSV-2 replication is at genital sites with colonization of the sacral ganglia. In animal models, sex hormones modulate susceptibility to HSV-2, with estradiol, but not progesterone, delaying vaginal pathology. In human females, infection is manifested by vesicles on the mucous membranes of the labia and the vagina (Fig. 4D). Severe forms result in ulcers that cover the entire area surrounding the vulva (Fig. 4B). Symptoms of primary infection include itching, pain, and lymphadenopathy. Systemic symptoms are more common in women than men. They often accompany the appearance of primary lesions and include fever, headache, photophobia, malaise, and generalized myalgias. Dysuria, urinary retention, urgency, and frequency, pain, and discharge are also seen. As a rule, they are not seen in recurrent disease. Cervical involvement is common, although it generally passes unnoticed (Fig. 4E), and the patient is often mistaken for having asymptomatic shedding. It likely contributes to the development of abnormal Papanicolaou smears. HSV infections of the genitourinary tract in women are easily mistaken for more common urinary tract infections, as the major symptoms—dysuria, urinary retention, urgency, and frequency, pain, and discharge—are similar. Therefore, when a patient presents with these symptoms, particularly in the absence of typical HSV lesions in the vulvar, vaginal, or cervical regions, cultures should be taken for both HSV and common gram-negative organisms. Common sites of primary HSV-2 infection in males are the shaft of the penis, the prepuce, and the glans penis (Fig. 4A). Urethritis is the main local expression accompanied by a watery discharge, often resulting in dysuria. Symptomatic urethritis is rare in recurrent disease, but virus can often be cultured from the urethra. Anal and rectal infections are seen primarily in homosexuals, as a result of anal intercourse. Atypical genital herpes is often described in immunocompromised patients and can present as large hyperkeratotic ulcers. An unusual HSV-2 presentation takes the form of large hyperproliferative lesions (Color Plate 5A) that may be caused by acyclovir-resistant virus, are associated with ICP10PK overexpression, and are difficult to treat (Beasley et al., 1997). In AIDS patients, HSV can produce persistent mucocutaneous disease.

Immunocompromised adults can develop severe generalized disease that is occasionally responsible for herpetic hepatitis. HSV pneumonitis accounts for 6 to 8% of cases of interstitial pneumonia in recipients of bone marrow transplants. The mortality rate due to HSV pneumonia in immunosuppressed patients is >80%. Generalized HSV with involvement of adrenal glands, pancreas, small and large intestine, and bone marrow was reported in the immunocompromised patient. HSV has also been isolated from 40% of patients with acute respiratory distress syndrome. HSV-1 DNA was recently found by PCR in gastrointestinal sensory neurons and in the geniculate ganglion and adjacent areas, respectively implicating HSV in recurrent gastrointestinal disorders and Bell's palsy. HSV-1 seropositivity was also associated with myocardial infarction and coronary heart disease in older adults, and Alzheimer's disease is believed to result from frequent episodes of mild encephalitis and neuronal apoptosis caused by virus reactivation in individuals homozygous or heterozygous for ApoE4 (Itzhaki, 2004). In immunocompromised patients, generalized HSV with involvement of adrenal glands, pancreas, small and large intestine, and bone marrow was reported. Severe generalized disease that is occasionally responsible for herpetic hepatitis also was described. Uncommon complications of HSV infection include monoarticular arthritis, adrenal necrosis, idiopathic thrombocytopenia, and glomerulonephritis.

HSE is the most commonly reported viral CNS infection, accounting for 10 to 20% of all cases. In children and young adults, HSE usually results from a generalized primary infection and is generally due to HSV-1. HSV-2 is involved in approximately 4 to 6% of cases. Both serotypes can cause encephalitis in infants. With treatment, HSE mortality rates are 15% in newborns and 20% in others. Survivors often have neurological sequelae involving impairments in memory, cognition, and personality. Untreated, mortality rates are 60 to 80%. Virus presumably enters the CNS through neurotropic spread by way of the olfactory bulb and can be isolated from other organs in addition to the CNS. A level of uncertainty exists as to the contribution of reinfection with exogenous virus versus reactivation of endogenous (latent) virus. The recent finding that HSV-1 can establish CNS latency and is subject to reactivation (Chen et al., 2006) argues in favor of the latter. Mouse studies implicated TK and $\gamma_1$34.5 in HSV-1 growth in the CNS after intracerebral inoculation and its invasion after peripheral inoculation (neurovirulence). HSE also has an apoptotic component associated with Jun N-terminal protein kinase (JNK)/c-Jun activation that is apparently mediated by ICP0 (Perkins et al., 2003a; Sanfillipo and Blaho, 2006).

HSV-2 is a common cause of aseptic meningitis. It also causes benign recurrent meningitis or Mollaret's meningitis and can occur in the absence of a prior clinically overt episode (reviewed in Tyler, 2004). HSV reactivation from geniculate ganglia is the most important cause of Bell's palsy, an idiopathic peripheral facial paralysis of sudden onset that accounts for >50% of all cases of facial paralysis (Schirm and Mulkens, 1997). HSV DNA was found in monofocal epilepsy seizure epicenters (Sanders et al., 1997), and HSV-1 and HSV-2 were associated with a form of acute necrotizing myelitis, generally with an ascending pattern (Nakajima et al., 1998). Necrotizing HSV encephalitis was implicated in progressive dementia syndrome (Zachhuber et al., 1995). HSV DNA also was found in gastrointestinal sensory neurons, suggesting that HSV could be involved in recurrent gastrointestinal disorders and peptic ulcer (Lohr et al., 1990). A high proportion (45%) of biopsy specimens from coronary arteries of patients undergoing coronary artery bypass grafting were positive for HSV-2, but not HSV-1,

antigen, associating HSV-2 infection with atherosclerosis (Raza-Ahmad et al., 1995). HSV antigen and DNA were seen in spermatozoa (Color Plate 6C), especially in primary infection, but also in asymptomatic subjects and may be associated with infertility (L. Aurelian, unpublished data; Kotronias and Kapranos, 1998). An unusual aspect of HSV pathogenicity is the infection of epithelial basal and stem cells (Color Plate 6A and B) and $CD34^+$ bone marrow-derived stem cells (Ono et al., 2005). Potential risks associated with stem cell infection require better elucidation, an area of current research interest.

HSV Infection During Pregnancy and Neonatal Disease

Localized genital infection is the most common form during pregnancy, but rare visceral involvement, with approximately 50% mortality, was reported. Fetal deaths occurred in 50% of cases and did not correlate with the death of the mother. The rate of maternal primary infection prior to 20 weeks of gestation is 0.5 to 10% per year (Brown et al., 1991), and it is associated with spontaneous abortion in 25% of infected women. Fetal infection is generally due to virus shed at the time of delivery. The incidence of HSV-2 shedding at delivery is 0.01% to 0.6%, irrespective of past history and time of gestation. Most women whose children are infected (60 to 80%) are asymptomatic at the time of delivery and have neither a past history of genital HSV nor a sexual partner reporting genital infection. The incidence of cervical shedding in asymptomatic pregnant women averages 3%. In a recent national cross-sectional sample of pregnant women, the overall seroprevalence was 63% HSV-1, 22% HSV-2, and 13% both serotypes. Seroprevalence differed with race and ethnicity, with non-Hispanic whites more likely to be seronegative than the other groups (40% versus 11%; $P < 0.001$). The rate of neonatal herpes projected by this study was 33 of 100,000 live births, and it was highest in the seronegative women (Xu et al., 2007a). Neonatal infection is decreased by surgical abdominal delivery when membranes are ruptured for less than 4 h. Intrauterine infection causing abortion or stillbirth as well as skin scars (cutaneous manifestations), ophthalmologic findings (chorioretinitis, microphthalmia), and neurological involvement (brain damage) is also possible but relatively rare. Often, it is associated with ascending infection in women who have had prolonged rupture of membranes before delivery. However, HSV can cross the placenta and cause placental infection manifested by deciduitis and villitis. These placental pathological changes may increase fetal damage (Duin et al., 2007). For women with a past history of genital infection, PCR testing of genital secretions can establish whether virus is shed at the time of delivery.

Neonatal HSV infections may remain localized to the site of infection (viz., skin, eye, or mouth), extend to the CNS, or disseminate to multiple organs. Neonates have the highest frequency of visceral and CNS involvement of all HSV-infected patients. Skin lesions are the most commonly recognized features of the disease, but disseminated disease is also seen in at least 70% of untreated cases. Factors influencing the extent of HSV disease in the neonate include maternal and host immunity, virus load, site of inoculation, and virus strain. The most significant predictor of mortality and morbidity in neonates is disease severity. Babies with the most severe disease (defined by level of consciousness or development of pneumonia) have the worst outcome. Clinical diagnosis of genital HSV infection during pregnancy is a significant independent predictor of perinatal HIV transmission. HSV suppression during pregnancy may be a strategy to reduce perinatal HIV transmission.

Latency

Latency is the hallmark of HSV infections. It is defined as virus persistence in the infected host in a repressed state that is compatible with cell survival and is often accompanied by episodes of virus reactivation and clinical symptoms. The pathogenesis of latency poses the following questions: (i) what tissues harbor latent infection, (ii) how is latency established and maintained, (iii) what mechanisms underlie virus reactivation, and (iv) what mechanisms regulate the development of clinical recurrences? Most of the available information regarding these questions was obtained from studies of animal models.

What Tissues Harbor Latent Virus?

Following replication in the skin, sensory or autonomic nerve endings are infected by cell-to-cell spread and virus capsids are transported intra-axonally to the nerve cell bodies (retrograde transport), where viral DNA is maintained as an episome that is largely transcriptionally silent (Fig. 5). However, maintenance and growth of the neural cells in tissue culture results in the production of infectious virus and subsequent permissive infection of susceptible cells—a process called cocultivation. Using this process, trigeminal and sacral dorsal root (S2 to S4) ganglia were respectively identified as the most common sites for latent HSV-1 and HSV-2 infections, but latency can also be established in the CNS, primarily in the brain stem (Chen et al., 2006). Real-time PCR of 970 individual sensory neurons obtained by laser-capture microdissection (LCM) from 5 subjects (at autopsy) indicated that 2 to 10.5% were positive for HSV-1 gG DNA, with a median of 11.3 copies/positive cell. This compares to 0.2 to 1.5% of neurons found positive for HSV-1 LAT by in situ hybridization (ISH), suggesting that HSV-1 infection is more pervasive than previously believed. In fact, combined ISH-LCM-PCR indicated that the majority of the latently infected neurons did not accumulate LAT to detectable levels. Persistence is almost exclusively in neurons, with only 21 copies of HSV-1 DNA found in approximately 5,200 nonneuronal cells collected by LCM (Wang et al., 2005). Ganglia can be colonized by multiple HSV strains, as determined by restriction endonuclease and restriction fragment length polymorphism studies (Buchman et al., 1979; Umene et al., 2007). This is particularly relevant for the management of the patient with drug-resistant infection, who may only respond to treatment during certain recurrent episodes. In one patient with acyclovir-resistant HSV-1, TK mutations were found in 11 of 12 studied ganglia, with clonal frequencies of 4.2 to 76% of mutants per ganglion. When individual neurons collected by LCM from one ganglion were analyzed by PCR, 6 of 14 neurons were coinfected with TK mutants and wild-type virus, 4 of 14 were infected with wild-type virus alone, and 4 of 14 were infected with TK mutants alone. The data suggest that diverse TK mutants arise independently under drug selection and establish latency in human sensory ganglia alone or together with wild-type virus (Wang et al., 2007).

During acute infection, 7 to 10% of HSV-1 or HSV-2 antigen-positive sensory neurons carried the A5 marker and 13 to 16% carried the KH10 marker, a representation similar to that in uninfected mouse trigeminal ganglia. However, during latency, HSV-1 LAT was seen in 25% of A5 neurons and 12% of KH10 neurons, while HSV-2 LAT was seen in 4% of A5 neurons and 42% of KH10 neurons.

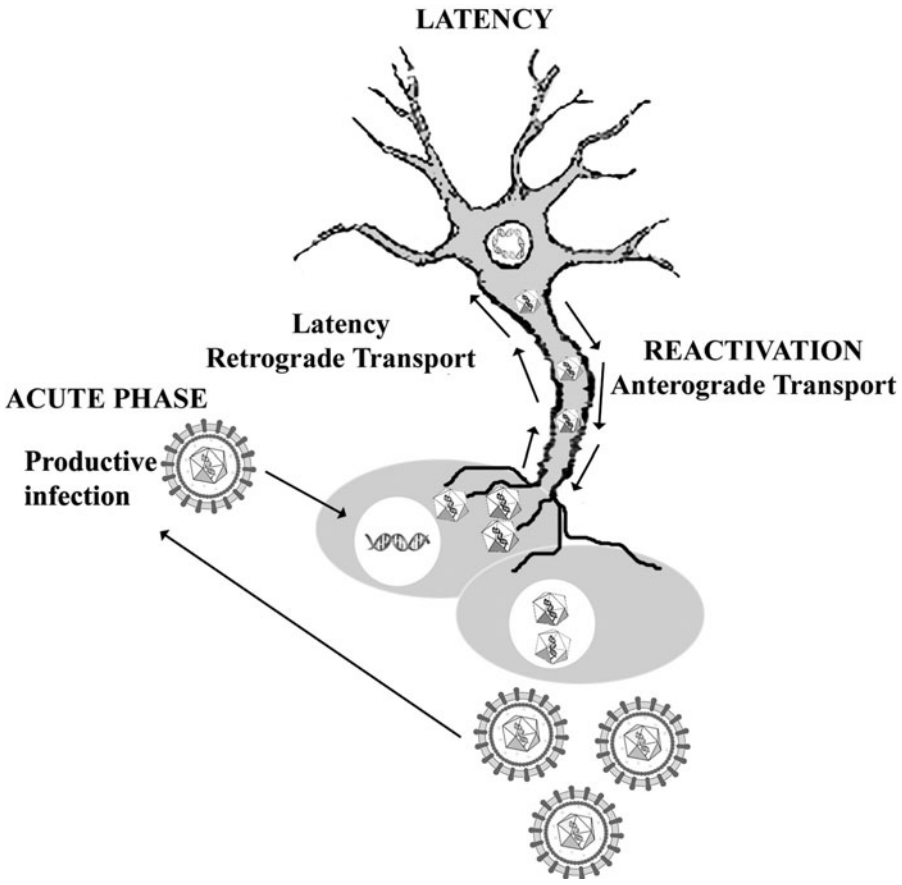

FIGURE 5 Schematic representation of latency establishment and reactivation. Following replication in the skin, capsids are axonally transported to nerve cell bodies where viral DNA is maintained as a circularized episome. Reactivating stimuli cause reverse axonal transport of virus progeny.

A chimeric HSV-2 mutant that expresses the HSV-1 LAT exhibited an HSV-1 phenotype (preferentially establishing latency in A5-positive neurons), suggesting that the HSV-1 and HSV-2 LAT regions influence the ability of the virus to establish latency in different neuronal subtypes (Margolis et al., 2007). In mice, HSV-1 DNA was detected by PCR also in the epithelial cells of the cornea, a finding interpreted to reflect active persistent infection (Maggs et al., 1998). However, DNA detection by PCR may reflect delayed clearance rather than persistent infection, as evidenced by the presence of HSV DNA in the skin from HAEM patients for 3 to 5 months after lesion resolution but not at 11 to 13 months, when it was ultimately lost (reviewed in Aurelian et al., 2003, and Aurelian and Burnett, 2008).

How is Latency Established and Maintained?

The number of latently infected neurons and the rate of spontaneous recurrences are proportional to the titer of the input virus and its amplification through efficient replication at the periphery (Lekstrom-Himes, 1998). However, mutants that cannot replicate in any cell in vivo can also establish and maintain latency, suggesting that replication at the periphery is not essential for latency establishment (Katz et al., 1990). Unenveloped capsids are transported by retrograde axonal transport from the periphery to the nerve cell bodies in the ganglia that innervate the site of infection. In humans, the time between peripheral infection and virus spread to the ganglia is unknown. In animal models, HSV-1 is recovered from trigeminal ganglia within 1 to 2 days after peripheral inoculation, associated with a short period of virus replication that may be determined by a conserved region in ICP4 (Bates and DeLuca, 1998). It is unclear whether ganglionic replication occurs in humans. At most, 2 to 4 weeks after resolution of the primary disease, latency is established and viral DNA is maintained as an episome that is largely transcriptionally silent.

The mechanisms by which latency is established and maintained are still unclear and may involve viral or cellular genes. Viral genes include TK and the overlapping gene UL24 (Jacobson et al., 1998), ICP0 (Wilcox et al., 1997), and VP16 (Tal-Singer et al., 1999). However, data interpretation is complicated by the finding that mutations that decrease the efficiency of virus replication have a negative impact on the ability of the virus to establish latency and reactivate (Hay et al., 1995). The only HSV transcript abundantly expressed during latency is the 2.0-kb intron derived from the 8.3-kb primary LAT. Because LAT overlaps the 3′ end of ICP0, it was originally proposed that it acts as antisense to ICP0. However, the absence of LAT does not allow ICP0 expression, and LAT mutants establish latency (Javier et al., 1988) and two ORFs (ORF-O and ORF-P), which are read antisense to LAT on the HSV genome, may

have contributed to the phenotype of the mutants used to examine LAT's function. An alternative hypothesis was that ICP0 splicing and expression are suppressed by the ORF-P protein (apparently encoded by a minor LAT). However, both spliced and intron-containing ICP0 transcripts were found in latently infected ganglia, and LAT or ORF-P did not increase their levels. One group has reported the expression of a LAT protein encoded by an ORF within the 2.0-kb intron which enhances lytic genes and promotes interaction with cellular transcription factors (Thomas et al., 2002). Recent studies have shown that HSV-1 LAT encodes a micro-RNA that may contribute to HSV persistence by modulating transforming growth factor β (TGF-β) signaling (Gupta et al., 2006). However, the function of the micro-RNA in sensory neurons is still unclear.

Cellular genes that may be involved in latency establishment and maintenance include an HCF-binding protein, known as Zhangfei, that is selectively expressed in human sensory neurons and inhibits IE gene expression by associating with VP16 and inhibiting formation of the VP16/HCF/oct-1 complex on TAATGARAT motifs (Akhova et al., 2005). The neural F-box 42-kDa protein (NFB42), a component of the E3 ubiquitin ligase that is specifically expressed in the brain, may also contribute to latency establishment by binding to the phosphorylated UL9 protein, causing its export to the cytosol, where it is degraded, thereby preventing active DNA replication (Eom et al., 2004).

There is a long-held belief that the immune system controls virus replication, thereby contributing to latency maintenance. The detection of T cells and cytokines in latently infected human trigeminal ganglia supports this interpretation. However, it is still unclear whether the T cells in the ganglia are antigen driven, in terms of both their recruitment and retention, nor is it clear how they may contribute to sustain latency. Trigeminal ganglia latently infected with HSV-1 were shown to contain a high number of activated ($CD69^+$) $CD8^+$ T cells that are oligoclonal and clustered around the latently infected neurons, but only a relatively small proportion were virus specific and expressed the cytolytic molecule granzyme B, which had previously been implicated in their ability to inhibit HSV reactivation. T-cell infiltration could reflect latency maintenance or an immune response to the initiation of virus replication. However, latency-induced changes are pivotal for T-cell recruitment because the T cells were not seen in ganglia from mock-infected mice (van Lint et al., 2005; Verjans et al., 2007).

How Does Virus Reactivate?

Certain stimuli cause reactivation of latent virus with concomitant reverse axonal transport of virus progeny to a peripheral site, at or near the portal of entry (anterograde transport) (Fig. 5). According to one model, unenveloped capsids are transported on axonal microtubules separately from vesicles containing glycoproteins, and assembly of enveloped virions occurs at axon termini by budding of capsids into membrane vesicles that contain the viral glycoproteins. The alternative model suggests that capsids acquire an envelope containing glycoproteins in neuronal cell bodies and travel in axons as enveloped virions (Snyder et al., 2007). Reactivating stimuli include fever, axonal injury, exposure to UV irradiation (sunlight), emotional stress, and hormonal irregularities. In the guinea pig model, latent virus reactivates spontaneously. In other animal models, virus is reactivated by neurectomy, ganglionic trauma, electrical stimulation, epinephrine iontophoresis, cadmium treatment or ganglia explantation, and organ culture. Spontaneous reactivation in humans is induced by exposure to local or systemic stimuli. Local stimuli include injury to tissues innervated by latently infected neurons (viz., sunlight). Systemic stimuli include fever due to bacterial or viral infections, menstruation, physical or emotional stress, and hormonal imbalance. They can cause simultaneous reactivation of virus latent in the trigeminal (HSV-1) and sacral (HSV-2) ganglia. The genome copy number in latently infected ganglia (which is determined by the rate of virus replication at the site of infection) is an important parameter for HSV reactivation (Maggioncalda et al., 1996; Lekstrom-Himes et al., 1998).

The role of specific viral genes in reactivation is still unclear. Latently infected ganglia express the 2.0-kb LAT and a spliced derivative of the 2.0-kb transcript that is about 1.5 kb and seems to be required for HSV-1 reactivation in the rabbit ocular model (reviewed in Jones, 2003). Dysregulated expression of an ORF contained within the 2.0-kb LAT was shown to enhance virus growth presumably by substituting for deficiencies in IE gene expression by a protein-dependent effect (Thomas et al., 2002), but the existence of a LAT protein is controversial. Most studies attribute the role of LAT in latency reactivation to its antiapoptotic activity, which ensures a large pool of neurons that can sustain replication of the reactivated virus. However, controversy remains. While LAT enhances reactivation in the rabbit eye model, it does not seem to be required in small-animal models. LAT antiapoptotic activity was not studied in neurons, although apoptosis is cell type-specific, and it may be unique to certain HSV-1 strains. Mutants lacking LAT were shown to reactivate as well as the wild-type virus. A recent study used an HSV-1 mutant in which 20% of the primary 8.3-kb LAT transcript (which is sufficient for enhancing the reactivation phenotype) was deleted and the baculovirus cIAP antiapoptotic gene was introduced under the control of the LAT promoter. This mutant was shown to have a wild-type reactivation phenotype in the mouse trigeminal ganglion explantation model (Jin et al., 2007), suggesting that the LAT antiapoptotic activity is sufficient to account for its function in latency. Indeed, stress-induced cyclic AMP early repressors caused LAT downregulation and apoptosis. Cytokines may initiate or contribute to LAT-mediated virus reactivation through interaction with STAT1 response elements in the LAT promoter (Kriesel et al., 2004).

HSV-1 reactivates more efficiently than HSV-2 from trigeminal ganglia, while HSV-2 reactivates more efficiently than HSV-1 from lumbosacral dorsal root ganglia. This ganglionic specificity correlates with the levels of DNA, with HSV-1 DNA being more abundant in the lumbar spinal cord and HSV-2 DNA in the sacral spinal cord (Bertke et al., 2007). LAT-influenced latency establishment in specific neuronal subtypes could be an important part of the mechanism by which LAT influences the viral reactivation phenotype, because a chimeric HSV-2 mutant that expresses the HSV-1 LAT has a characteristic HSV-1 reactivation phenotype (Margolis et al., 2007). LAT is not involved or, at best, is an inefficient and weak determinant of spontaneous HSV-2 reactivation in the guinea pig model and the HSV-2 LAT fails to substitute for its HSV-1 counterpart in promoting latency reactivation (Wang et al., 1997). By contrast, an HSV-2 mutant with a deletion of ICP10PK was significantly impaired in latency establishment and reactivation, and virus reactivation from the rare ganglia in which latency was established was inhibited by an ICP10 antisense oligonucleotide, suggesting that ICP10PK is involved in HSV-2

latency (Smith et al., 1992; Smith et al., 1998; Aurelian et al., 1999; Aurelian and Smith, 2000; Wachsman et al., 2001). Indeed, ICP10PK is required for optimal IE gene expression and virus growth, notably in neurons (Smith et al., 1998; Perkins et al., 2002; Laing et al., 2006).

We proposed that ICP10PK is involved in HSV-2 reactivation in response to AP-1 upregulation by latency-reactivating stimuli (Fig. 6). The ICP10 promoter is the only viral promoter with two functional AP-1 *cis* response elements. Basal expression from this promoter is AP-1 dependent (Zhu and Aurelian, 1997), and it responds to AP-1 upregulated by latency-reactivating stimuli (Gober et al., 2005). During reactivation, R1 transcripts are detected before IE transcripts (Tal-Singer et al, 1997), presumably related to the role of ICP10PK in IE gene expression (Smith et al., 1998). An AP-1 amplification loop is generated by ICP10PK through activation of the Ras signaling pathway, which culminates in increased AP-1 expression (Smith et al., 2000). The ribonucleotide reductase activity of the newly synthesized ICP10 also allows viral DNA synthesis in neurons. Reactivating stimuli also cause nuclear translocation of HCF, which is required for VP16-mediated IE gene activation (Kristie et al.,

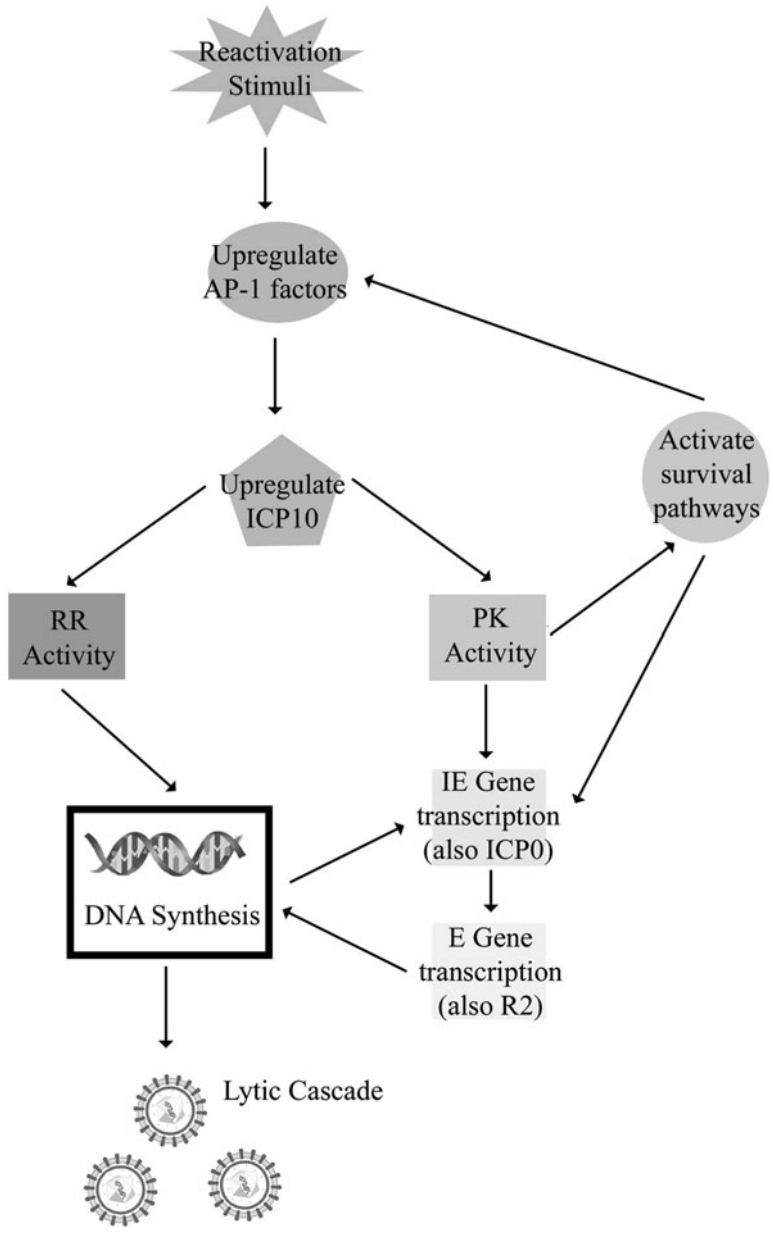

FIGURE 6 Proposed mechanism of HSV-2 latency reactivation. Reactivating stimuli upregulate AP-1 factors, thereby causing ICP10 overload. ICP10 supplies the PK activity, which is required for IE gene transcription and a feedback amplification loop through activation of the Ras survival pathway. It also supplies the ribonucleotide reductase (RR) activity that is required for DNA synthesis. The outcome is initiation of the lytic cascade.

1999). The outcome is initiation of the lytic cascade and the production of infectious virus. However, the potential relationship between ICP10PK and LAT in HSV-2 latency and reactivation, if any, is still unclear. An alternative, but not mutually exclusive, interpretation is that reactivating stimuli downregulate or functionally impair cellular proteins (viz., octamer protein oct-2) that inhibit viral gene expression in latently infected ganglia (Latchman, 1994).

What Determines Disease Causation?

Only some reactivation episodes result in clinical disease. As shown by us over 20 years ago and confirmed by many others since that time, the development of clinically apparent recurrent lesions is associated with robust replication of the reactivated virus resulting from the avoidance of immune-mediated containment. Longitudinal studies of HSV-1 and HSV-2 patients indicated that clinical lesions are associated with unimpeded replication of reactivated virus resulting from transient downregulation of HSV-specific T-cell responses. This decrease first occurs during prodrome, at 1 to 2 days before lesion onset, and lasts throughout the acute episode. It is due to a shift in the balance of T helper populations, in favor of the IL-6- and IL-10-producing Th2 cells, and was associated with a population of T regulatory (suppressor) cells. Patients with UV-induced HSV recurrences evidenced decreased levels of HSV-specific T-cell proliferation and increased levels of Th2 cells that produce IL-6. Proliferation was restored by IL-6 neutralization, indicating that Th2 cells are involved in the immune downregulation associated with UV-induced recurrent disease (Miura et al., 1994). IL-6 production also was increased in latently infected ganglia from UV-treated mice, while Th1 lymphokines IFN-γ and IL-2 were decreased. T regulatory cells, previously discredited as a mechanism of immune regulation, are again gaining support. They include $CD4^+$ $CD25^+$ cells, which function via cell-to-cell interaction and the production of TGF-β, and Tr1 cells, which function via secretion of TGF-β and IL-10 and are consistent with our earlier reports. Keratinocytes infected by the reactivated virus can induce immune suppression by shifting the profile of cytokine production towards Th2 (reviewed in Aurelian, 2004).

Neoplastic Transformation

Seroepidemiologic studies have associated HSV-2 infection with an increased risk of human squamous cervical cancer. Current opinion is that HSV-2 is a cocarcinogen. A wealth of experimental evidence indicates that HSV-2 is a tumor virus. It causes neoplastic transformation of immortalized cells (including those of human origin) and causes tumors in animals, both under conditions that interfere with virus replication. Transformation is mediated by ICP10PK through activation of the Ras/MEK/ERK (extracellular signal-regulated kinase) pathway. ICP10PK activates Ras by inhibiting the negative regulator RasGAP through phosphorylation and by binding the Grb2/SOS complex, thereby favoring interaction of Ras with the guanine nucleotide exchange factor SOS (Aurelian, 1998; Smith et al., 2000; Aurelian and Smith, 2001; Smith, 2005). In neurons, which are nonreplicating differentiated cells, ICP10PK-mediated activation of the Ras/MEK/ERK pathway, is associated with survival from toxic insults (Fig. 7). The HSV-1 R1 gene (also known as ICP6) did not conserve the PK activity and cannot impart neuronal survival (Fig. 7). It is also not oncogenic in immortalized cells. However, HSV-1 causes mutagenesis and gene amplification. In humans, HSV-2 can cause severe hyperproliferative lesions that are associated with increased production of ICP10PK (Color Plate 5) and may have an increased risk for neoplastic conversion (Beasley et al., 1997).

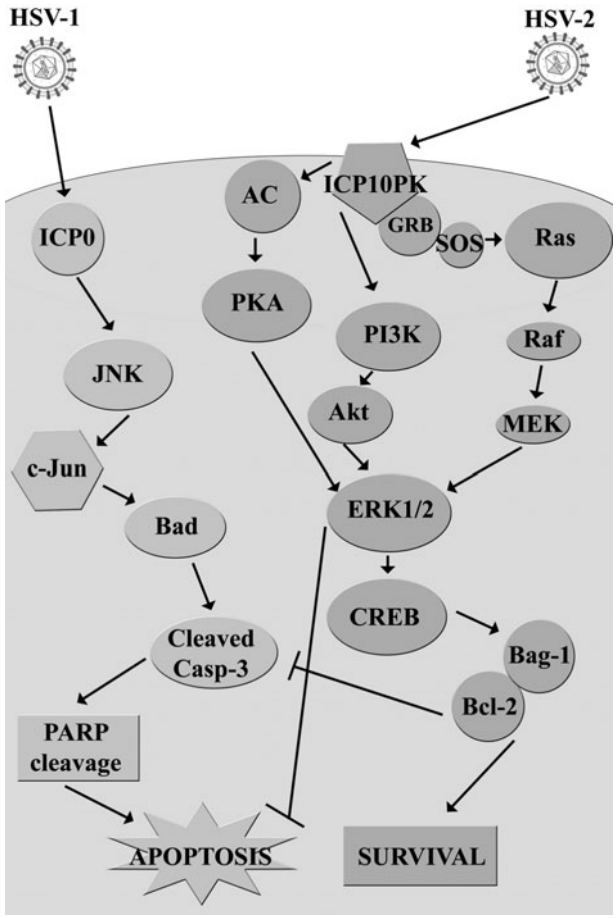

FIGURE 7 HSV-1 and HSV-2 activate distinct signaling pathways related to pathogenicity. Schematic representation of signaling pathways activated by neuronal cell infection with HSV-1 or HSV-2. HSV-1 activates the proapoptotic JNK/c-Jun pathway and triggers apoptosis, likely mediated by the ICP0 protein. HSV-2 activates the MEK/ERK, phosphatidylinositol 3-kinase (PI3K)/Akt, and adenylate cyclase (AC)/PKA survival pathways and blocks apoptosis, mediated by the PK function of the R1 protein (also known as ICP10). PARP, poly(ADP-ribose) polymerase.

DIAGNOSIS

Clinical, Histopathology, and Cytology

Clinical diagnosis is still routinely used. Histopathologic changes are a combination of virus-induced alterations (cell ballooning, condensed nuclear chromatin, and nuclear degeneration) and associated inflammatory responses. Virus-induced changes are generally within the parabasal and intermediate cells of the epithelium. Multinucleated giant cells are also formed, and a clear (vesicular) fluid containing large quantities of virus, cell debris, and inflammatory cells appears between the epidermis and the dermal layer. An intense inflammatory response is seen in dermal structures, particularly in primary infection, and is accompanied by an

influx of mononuclear cells. Perivascular cuffing and areas of hemorrhagic necrosis also are seen in the area of infection, particularly in organs other than skin. In the brain, oligodendrocytic involvement, gliosis and astrocytosis are common. Polykaryocytosis is detected by cytologic examination of tissue scrapings (Zanck assay). However, its insensitivity argues against the use of cytology at a time when other, equally rapid tests are available. In children and adolescents (approximately one-third of cases), HSE diagnosis is suggested in the encephalopathic, febrile patient with focal neurologic signs. However, these clinical findings are not pathognomonic because numerous other CNS diseases can mimic HSE. Neurodiagnostic evaluation can provide support for the diagnosis by the demonstration of temporal lobe edema or hemorrhage by magnetic resonance image scan and spike and slow-wave activity on electroencephalogram.

Virus Culture

Sensitivity, specificity, speed, and availability determine the choice of a laboratory test for HSV diagnosis (Table 3). The presence of viable (infectious) virus in a clinical specimen is the definitive evidence of HSV infection (gold standard). HSV can be isolated from oral and genital lesions, ocular samples, throat swabs and washings, bronchial or alveolar washings, biopsy specimens from the brain and other tissues, and CSF. CPE can be seen within 18 h p.i., but it generally takes 2 to 3 days (depending on the sensitivity of the cells and the amount of virus inoculated). Unfortunately, cultures are routinely kept for 14 days, and this is a major limitation when rapid diagnosis is essential, such as in HSE or systemic diseases, where therapy must be initiated early. Sample collection is a major factor in determining the success of virus isolation. When a patient presents with skin vesicles, they should be punctured and the vesicular fluid taken on a swab. The base of the lesion also should be swabbed. For best results, specimens should be inoculated within 1 h after collection. Otherwise, they should be put in transport medium and refrigerated or frozen (at –70°C) to preserve the specimen. The transport medium routinely used in our laboratory is Eagle's minimal essential medium with Earle's salts and L-glutamine supplemented with 0.8% bovine serum albumin fraction V, 10 mM HEPES buffer and antibiotics (100 μg of penicillin-streptomycin/ml, 5 μg of amphotericin/ml). Primary cells are generally more sensitive than established lines. HSV causes a characteristic CPE, consisting of rounded refractile cells. If hematoxylin-eosin stain is applied, the cells will exhibit eosinophilic intranuclear inclusions. Polykaryocytosis depends on the virus strain and the cell type. Results may be confounded by the presence in the specimens of other viruses that cause a similar CPE, such as adenovirus, a common isolate in ocular specimens. To address this problem, at least two cell types that differ in their ability to support the growth of these viruses should be used. We use human lung fibroblasts (MRC-5) and lung carcinoma (A549) cells. HSV grows in MRC-5 cells; adenovirus does not. In A549 cells, adenovirus CPE is first seen on days 4 to 5; that of HSV is seen on days 1 to 3. Ultimately, however, virus identification can only be done using specific antisera in an appropriate immunologic test. Best virus culture results are obtained with specimens collected when the lesion is still in the vesicular or early ulcerative stage (days 1 to 3 postonset) at which time mean virus titers are significantly higher ($\log_{10}$ PFU ± standard error of the mean [SEM] = 2.5 ± 0.5) than at later times ($\log_{10}$ PFU ± SEM = 1.3 ± 1 on day 4 postonset).

Antigen Detection

Antigen detection provides an alternative approach to virus culture, which has the advantage that it is rapid, sensitive (identifies nonviable virus), and relatively inexpensive. Antigen in the lesion interacts with the added HSV antibody, producing a complex that may be detected by means of a variety of methods, such as immunofluorescent (IF) or immunoperoxidase (IP) staining, or immunoassay (generally, enzyme-linked immunosorbent assay [ELISA]). Presently available purified or monoclonal antibodies to HSV proteins have improved the specificity of these assays and provide the necessary tools to differentiate between the two HSV serotypes (based on glycoprotein gG). Direct assays employ HSV antibody conjugated to a detector molecule. Indirect assays use unconjugated HSV antibody followed by the appropriate conjugated anti-immunoglobulin. To amplify the signal, anti-HSV antibody conjugated to biotin is added to the culture, followed by the addition of avidin-fluorescein conjugate. In direct comparison, we found that the sensitivity of the IF assay is 70% and 53.8% of that of

TABLE 3 Methods for the diagnosis of HSV infections

Type of infection	Method	Advantage(s)	Disadvantage(s)	Cost	General availability
Skin, mucocutaneous	Isolation	High sensitivity/specificity	Slow; complex	High	Variable
	Cytology (Tzanck)	Rapid, simple	Poor-fair sensitivity/specificity	Low	Yes
	Antigen				
	IF; IP	Good sensitivity, rapid, simple	Poor-fair specificity	Low	Yes
	ELISA	Good sensitivity, rapid, simple	Inconvenient		
	Shell vials	Better sensitivity/specificity	Slower than direct	Low/high	Yes
Keratitis	Isolation	High sensitivity/specificity	Slow, complex	High	Variable
	Antigen detection				
	IF; IP	Good sensitivity, rapid	Poor-fair specificity	Low	Yes
Encephalitis	PCR	Highest sensitivity, rapid	Potential specificity problems	Medium	Variable
Previous infection	Serology				
	Increased titers	Good sensitivity/specificity	Only primary infection	Medium	Variable
	Serotype	Good specificity/sensitivity	Variable results	Medium	Variable

virus culture when specimens are collected from primary and recurrent lesions, respectively. This correlates with mean titers of total recoverable virus ($\log_{10}$ PFU ± SEM) of 4.0 ± 1.0 and 2.5 ± 0.5, respectively, in primary and recurrent lesions. Antigen detection is more effective in diagnosing ocular HSV infection. The cornea is anesthetized, and the corneal epithelium is scraped with a sterile scalpel blade and transferred to sterile phosphate-buffered saline on glass slides. The blade is washed in transport medium, and a swab of the affected area is placed in virus transport medium. Standard procedures for indirect IF are followed to obtain a preliminary diagnosis while awaiting confirmation from virus culture.

IP has the advantage that it requires an ordinary light microscope and the slides can be kept indefinitely. IF has the advantage that its sensitivity has been greatly increased by the use of confocal microscopy. However, both procedures suffer from specificity limitations, particularly in the presence of vesicle fluid, mucus, or other protein-binding secretions. Factors that affect data interpretation include tissue and cell fixation, duration and type of antigen retrieval, antibody specificity, and dilution and detection systems. Cutoff levels for assessing whether a tissue is "positive" or "negative" can vary even for the same antigen. Quality assurance schemes for the methodology have improved the standards of staining. However, errors in data interpretation can occur. Reproducible, standardized, and easy to use protocols are needed for immunohistochemistry quantification. Improvements in automated analysis with wider applicability could lead to standardization (Walker, 2006). The current routine procedure increases the sensitivity and specificity of the antigen detection assays by amplification through short-term (16 to 24 h) growth in tissue culture. This procedure is known as shell vial and in many laboratories as virus isolation, although infectious virus is not obtained. Specimens are inoculated onto cell cultures on coverslips in shell vials, and the cells are stained, generally by IF, 16 to 24 h later. Low-speed centrifugation (700 × *g*, 40 min, room temperature) of inoculated shell vials increases virus adsorption onto the cell monolayers, thereby enhancing the sensitivity of the assay. Known negative and positive cultures (uninfected and HSV-infected, respectively) are used as controls. A modification of the shell vial assay (suspension-infection) combines the specimen with suspended culture cells before allowing them to grow (Rich and Johnson, 1998). The sensitivity of the shell vial assay can be identical to that of virus culture or significantly lower (66.2%).

ELISA is another antigen detection assay. It is rapid, versatile, and adaptable to automation. The test specimen is directly attached to the wells of microtiter plates, or it is bound to antibody-coated wells. The wells are treated with HSV antibody and antiglobulin that is conjugated to an enzyme, such as alkaline phosphatase. Addition of substrate results in the formation of a soluble colored reaction product that can be read quantitatively in a spectrophotometer. Variables that affect the results include the quality of the HSV antibody, the buffer, the conditions used to bind the specimen to the plates, and the plastic used to make the plates. When compared to virus culture, the direct ELISA showed 97% sensitivity in nongenital specimens and 88% in genital specimens. The culture-amplified ELISA (4 days) showed a 95% sensitivity in all samples (Kok et al., 1998). A recent adaptation is the enzyme-linked virus inducible system (ELVIS) that employs a genetically altered BHK cell line, which allows for overnight HSV detection and simultaneous serotyping. The specimen is incubated overnight on shell vials containing these cells and stained with galactopyranoside (X-Gal). Blue cells mean that HSV is present. In direct comparison of ELVIS to shell vial culture and HSV detection with monoclonal antibodies, there was 96.1% agreement. After resolution of discrepancies, the sensitivity and specificity were 95.0% and 100%, respectively, for the shell vial and 95.0% and 98.6% for ELVIS (Crist et al., 2004).

Nucleic Acid Testing

ISH with radioactively labeled or biotinylated DNA probes can be used as a detection method for samples inoculated onto shell vials and can be as sensitive and specific as antigen detection or virus culture. PCR is an extremely sensitive and relatively simple assay that has been rapidly replacing standard HSV detection protocols. Commonly used primers are for DNA polymerase (Pol), UL42, gB, gD, or TK, and they target different sequences in these genes. Their sensitivity (viz., the number of detected HSV DNA copies) often varies 10- to 1,000-fold. Restriction digestion of the PCR products can be used for direct HSV typing. Assay modifications include (i) nested PCR, in which amplification is done twice, the second time with primers internal to those used in the first set (this is likely to increase sensitivity) and (ii) multiplex PCR, in which coamplification is simultaneously done with primers for various infectious agents. For example, multiplex PCR was described for HSV-1, HSV-2, and cytomegalovirus (Cassinoti and Siegel, 1998) and for HSV-2, *Haemophilus ducreyi*, and *Treponema pallidum* (Beyrer et al., 1998). However, the sensitivity of some primer sets can be artifactually decreased in coamplification, and this is generally difficult to control.

PCR is more sensitive than antigen detection and virus culture (Slomka et al., 1998). In our experience using Pol primers, the sensitivities of culture for detection of HSV-1 and HSV-2 were 0.20 and 0.61, respectively, compared with that of PCR. False-positive PCR results due to specimen contamination can be a major problem in a clinical setting that processes large numbers of specimens on a daily basis (Fomsgaard et al., 1998). To control for such problems, negative specimens must be interspersed between the clinical specimens, and the specificity of the amplificants must be confirmed by hybridization with specific (internal amplificant sequence) and unrelated (negative control) probes. Hybridization with the appropriate probe also increases the sensitivity of the assay, but it is relatively cumbersome, since it uses radioactivity and its turnaround is 2 to 4 days. As an alternative to hybridization, various commercial assays use colorimetric microtiter detection. The sensitivity and specificity of one such assay were 63% and 100%, respectively, when compared to hybridization. Two other commercial assays had 98.2 and 100% or 100 and 96.9% sensitivity and specificity, respectively (Tang et al., 1998). Data concordance between two centers was disappointing, but it was improved by DNA purification (Hirsch and Bossart, 1999).

Quantitative PCR is particularly helpful in HSE, where HSV DNA levels in CSF specimens have a wide variability, in order to evaluate response to antiviral drugs (<20 to 20,000 copies/ml). One such assay is a competitive PCR. Here, specimens are coamplified with an internal standard and the HSV/internal standard ratio is compared to a standard curve in which the same internal standard was coamplified with known amounts of HSV DNA. The assay can measure 10^0 to 10^6 copies of HSV DNA (Hobson et al., 1997). It was recommended that PCR be performed together with an immunoglobulin G (IgG) capture ELISA for HSV intrathecal antibody production to span the disease cycle from symptoms onset to

weeks after hospitalization (Fomsgaard et al., 1998). Guidelines issued by the International Herpes Management Forum regard PCR of the CSF as the choice method for HSE diagnosis, but negative results need to be interpreted in the context of clinical presentation and the timing of CSF sampling. CSF virus culture is of little value in all but those under 6 months of age, and CSF (intrathecal) antibody measurements are not recommended for acute diagnosis. It is helpful in retrospective diagnosis or in cases in which CSF is sampled late after onset of infection and PCR is negative. Real-time PCR is also the choice procedure for HSV DNA detection in the CSF from patients with primary and recurrent aseptic meningitis. In one study, using real-time PCR, HSV-2 DNA was found in CSF from 80% of the patients with clinical HSV-2 meningitis compared to 72% by nested PCR. The sensitivity of real-time HSV-2 PCR was 87% in primary meningitis, in which the virus load was higher, compared to 70% in recurrent meningitis, which had a lower virus load (Franzen-Rohl et al., 2007). However, the high sensitivity of the PCR has raised questions about the clinical significance of DNA detection in other tissues, such as epilepsy seizure centers (Sanders et al., 1997) or gastrointestinal sensory neurons (Lohr et al., 1990). Similarly, the proportion of HSV DNA-positive secretions from women in labor is about eight times higher than by culture methods (Cone et al., 1994), but the frequencies of HSV transmission to the infant and neonatal disease were not increased. Because the likelihood of infection depends on input virus load, a DNA-positive but culture-negative specimen may not be infectious. Alternatively, such specimens may contain DNA fragments rather than the full-length HSV genome, as is the case for HAEM lesions. To ensure that DNA positivity in a culture-negative specimen reflects the presence of low virus titers, PCR must be done with primers for genes that span the entire HSV genome and are equally sensitive (Aurelian et al., 2003).

Serology

Serology is not commonly used in virus diagnosis. It can be used to diagnose a primary infection in a patient presenting with clinically visible lesions, based on the demonstration of a significant rise (≥4-fold) in serum antibody levels over the course of the illness. This is done by taking paired serum samples. The first (acute phase) sample is preferably taken before 10 days (the time required to develop antibodies). The second (convalescent phase) sample is taken 2 to 3 weeks later. Patients with recurrent disease do not demonstrate increases in antibody levels. Serology can also be used as evidence of prior infection indicative of the need for prophylactic therapy (viz., in immunocompromised patients). Type-specific serodiagnostic techniques, which are currently available, are useful in evaluating past HSV-2 infections and in predicting the risk of recurrent disease, since genital HSV-2 is more likely to recur than genital HSV-1. Type-specific serologic assays use glycoprotein gG from HSV-1 (gG-1) and HSV-2 (gG-2), which are only 35% homologous, in immunodot and immunoblot strip methods, Western blotting, immunoblotting with baculovirus-expressed gG, and ELISA with gG purified on immunoaffinity columns. The Focus HerpeSelect HSV-1 and HSV-2 ELISA are licensed by the U.S. Food and Drug Administration. In sexually active adults, the sensitivities of these ELISAs were 91.2% (HSV-1) and 96.1% (HSV-2) and the specificities were 92.3% (HSV-1) and 97% (HSV-2) (HerpeSelect type-specific HSV-1 and HSV-2 IgG diagnostic kits; Focus Diagnostics). When these assays and Western blotting were used on sera from university students who reported no history of genital herpes, the seroprevalences of HSV-2 and HSV-1 were 3.4% and 48%, respectively, by Western blotting. The positive predictive value of the Focus HSV-2 ELISA was 37.5%, the sensitivity was 100%, and specificity was 94.1%. The positive predictive value of the Focus HSV-1 ELISA was 96.7%, the sensitivity was 69.0%, and the specificity was 97.8%. Thus, in this low-prevalence population, the positive predictive value of the Focus HSV-2 ELISA test was low. This finding, together with those reported elsewhere, indicates that caution is warranted when recommending HSV screening in low-prevalence or heterogeneous populations. Consideration should be given to raising the cutoff index value for defining a positive test result (Mark et al., 2007b) and using assays that are based on additional type-specific antigens. The use of a single antigen (gG) for type-specific serologic diagnosis suffers from a number of limitations, including loss of relevant epitopes during antigen preparation, preferential recognition of type-common immunodominant epitopes by sera from various individuals, variable timing of seroconversion to distinct epitopes, occurrence of gG-2-negative HSV-2 strains, variability of the gG-1 or gG-2 genes among wild-type virus strains, and loss of gG-1/gG-2 antibodies in a significant percentage of individuals. Indeed, gG-1-negative sera recognized type-specific peptides in the HSV-1 glycoprotein gC (Ackermann et al., 1998), and it is widely recognized that specificity and sensitivity can be improved by using assays based on type-specific epitopes in other HSV proteins (Schmid et al., 1999). ELISA using HSV-2- or HSV-1-specific R1 peptide antigens has high sensitivity and specificity (94.6% and 95.4%, respectively), determined by direct comparison to Western blotting, and can be used in conjunction with the gG-2-based assays to improve the sensitivity and specificity of serologic diagnosis and patient management (Wales et al., 2004).

IMMUNITY AND VACCINE DEVELOPMENT

Innate Immunity

An early nonspecific containment phase (innate immunity) contributes to protection. Natural killer (NK) cells and rapid production of IFN-I provide a threshold of resistance to HSV-1 infection and were associated with the natural resistance of certain mouse strains. In mice, IFN-α/β inhibits the onset of IE gene expression, limits virus spread into the nervous system, and activates NK cells. Dendritic cells (DC) are required for activation of NK cells as well as $CD4^+$ and $CD8^+$ T cells in response to HSV-1. Conventional $CD11c^+CD8\alpha^+$ DC are the principal antigen-presenting cells during acute HSV-1 infection. The plasmocytoid $CD11c^+$ $B220^+$ DC secrete large amounts of IFN-I in vitro after exposure to HSV-1 and help lymph node DC to optimally induce HSV-specific CTL during a primary immune response (Barr et al., 2007). ICP0 increases the susceptibility of HSV-infected cells to NK-mediated lysis dependent on natural cytotoxicity receptors (Chisholm et al., 2007). Toll-like receptors (TLRs) are single membrane-spanning noncatalytic receptors that recognize structurally conserved molecules derived from microbes collectively referred to as pathogen-associated molecular patterns (PAMPs) and play a key role in innate immunity. In HSV-2-infected subjects, two TLR2 haplotypes (2 and 4) were associated with increased rates of lesion development ($P = 0.008$ and $P = 0.03$, respectively) and shedding ($P = 0.02$ and $P = 0.001$, respectively), suggesting that polymorphisms in TLR2 may be in part responsible for differences in the severity of HSV-2 infection (Bochud et al., 2007).

Adaptive Immunity

HSV antibody is first seen on days 5 to 10 p.i. and persists indefinitely. Antibody titers are higher in subjects with a history of recurrent disease than in those without, reflecting virus reactivation. Development of antibodies to structural proteins is followed by antibodies directed against gD, gB, ICP4, gE, gG, and gC. IgM and IgG antibodies can be demonstrated at different times p.i. In children, antibodies to ICP4 may be predictive of long-term neurologic outcome. Infected newborns produce IgM antibodies (particularly against gD) within the first 3 weeks p.i. High titers of neutralizing antibody do not prevent reinfection or recurrent disease, nor do transplacentally acquired antibodies protect the newborn. T cells play the major role in recovery from HSV infection. Their development is delayed in newborns (2 to 4 weeks), presumably explaining the increased sensitivity to disease progression in this age group. T-cell effector mechanisms include: (i) delayed-type hypersensitivity, an early (days 5 to 10 p.i.) HSV type-common response that remains inducible for at least 2 years after infection; (ii) effector lymphokines, such as IFN-γ, IL-2, and other soluble factors; and (iii) CTL, specific for cell surface glycoproteins, primarily gD or gE, or intracellular IE proteins.

HSV has developed various immune evasion mechanisms. Notable among these is the inhibition of antigen presentation by the major histocompatibility complex class I through binding of the cellular protein that transports antigenic peptides into the endoplasmic reticulum by the viral IE protein ICP47. Other immune evasion mechanisms include resistance to attack by complement, mediated by viral glycoprotein gC, prevention of antibody binding, mediated by viral glycoproteins gE/gI, and blocking of $CD8^+$ CTL activity through U_S3-mediated inhibition of caspase activation. HSV-1 also blocks the IFN response at multiple sites, including decreased Jak1 kinase and Stat2, which are associated with IFN signaling (reviewed in Aurelian, 2004; Wiertz et al., 2007).

In the 1980s, we attempted to determine the role of immune evasion in recurrent disease by comparing the HSV-specific T-cell responses of infected subjects who do or do not develop recurrent disease. We found that T cells from patients who do not develop recurrent disease or are experiencing a lesion-free interval produce IFN-γ when stimulated with HSV antigen. By contrast, IFN-γ was not produced by T cells collected during recurrences or at prodrome (24 to 48 h before clinical outbreak). We called the IFN-γ-producing immune profile of patients who do not suffer from recurrent disease, "relevant immunity" to distinguish it from the immunity exhibited by patients with recurrent disease, which is an IL-6/IL-10-producing profile. Since that time, IFN-γ has been identified as a Th1 cytokine that has antiviral activity, induces $CD8^+$ CTL, and activates NK cells, while IL-6/IL-10 have been identified as Th2 cytokines that downregulate the Th1 response. Our studies implicated regulatory T cells (Tregs) in the immune downregulation that enables recurrent disease development (Aurelian, 1990). Following a relatively long interval during which Tregs had fallen into disrepute, they recently became again an area of major research interest, also in terms of their involvement in HSV recurrent disease, now in the mouse model (reviewed in Aurelian, 2004).

We proposed that onset of recurrent disease is regulated at two checkpoints. First is the decision for resumed transcription of latent HSV DNA, which is under the control of stress-induced cellular proteins, such as the AP-1 transcription factor, which activates the ICP10PK promoter, thereby initiating resumption of virus replication (Fig. 6). Second is the decision to allow unimpeded replication of the reactivated virus, which is under the control of newly synthesized viral protein(s) that are either Th2 polarizing and/or induce Tregs, thereby downregulating the antiviral Th1 responses. This interpretation predicts that a leak in the first, but not in the second, regulatory checkpoint would result in asymptomatic virus shedding, which we first documented in 1985 to 1990 (Aurelian and Kessler, 1985; Aurelian et al., 1990) and today is an established aspect of HSV pathogenesis. An additional prediction was that clinical recurrences are associated with the suppression of virus-specific Th1 responses, and this was validated by many studies, which also indicated that recurrent disease is associated with increased production of Th2 cytokines (Aurelian, 1990; Croft et al., 1994; Deshpande et al., 2000; Yeslikawa et al., 2000; McKenna et al., 2001; Singh et al., 2003).

Prophylactic Vaccines

Vaccines for HSV infection are not yet available for clinical use, but several approaches, described below, are currently in experimental and clinical trials. The original goals of vaccination were to induce mucosal and systemic immunity to prevent HSV-2 infection and transmission. Glycoproteins gD/gB, which are involved in cell entry, were considered the optimal targets. However, these goals were proven to be unrealistic and were replaced by prevention and/or reduction of clinical symptoms of primary infection. In this context, efforts focused on the selection of ideal adjuvants.

Subunit Vaccines

The advantage of the subunit vaccines is that they are safe, chemically defined, selective, and stable at ambient temperature. Their production is simple, easily standardized, and cost-effective. The most recently studied subunit vaccine is a gD-2 peptide formulated with a mixture of alum and 3-deacylated monophosphoryl lipid A as an adjuvant from GlaxoSmithKline. In two phase III trials, this vaccine reduced clinical symptoms of primary HSV-2 infection (approximately 70% efficacy) but only in women and only if they were HSV-1 and HSV-2 seronegative at the time of vaccination. The effect of gender is unlikely to be due to gender-specific immunological differences, because similar immunity was induced in males although they were not protected (Stanberry et al., 2002). The absence of efficacy in HSV-1-infected women suggests that previously established immunity may interfere with vaccination and predicts minimal impact on the proportion of the disease because HSV-1 infection is widespread. The vaccine did not protect from HSV infection, and its activity was attributed to the adjuvant used in its composition (Stanberry et al., 2002) because symptoms were not reduced when gD-2 was formulated with another adjuvant (Bourne et al., 2003).

Live Attenuated Virus Vaccines

Live attenuated virus vaccines have the advantage of imparting long-lasting and broad immunity. Their disadvantage is safety concerns related to the risk of homologous recombination during production in cell lines engineered to supply the absent viral gene product(s) in *trans*. One strategy to develop live HSV-2 vaccines used viral vectors that express HSV glycoproteins. Recombinant HSV-1 viruses expressing gD-2, gE-2, gG-2, and part of gI-2 were studied in animals and human clinical trials, with limited success. In animal models, the Oka vaccine strain of varicella-zoster virus or vaccinia virus recombinants expressing gD-2 reduced disease severity. These studies also demonstrated that glycosylation epitopes and DC-mediated antigen presentation

play determinant roles in protective immunity (reviewed in Aurelian, 2004.) More recently, *Listeria monocytogenes* expressing an H-2K^b gB-1 peptide was shown to induce a CD8$^+$ T cell response and protect from HSV-1 challenge in mice (Orr et al., 2007). Another strategy used to develop live vaccines was to generate mutants rendered nonvirulent by the deletion of one or more genes while retaining the viral glycoproteins previously identified as immunogenic targets. HSV-2 mutants with deletions of (i) both copies of the $\gamma_1$34.5 gene and the UL55 and UL56 ORFs, (ii) ICP8, or (iii) gH reduced disease severity and recurrent disease frequency in animal models. The virus with the gH deletion was studied in humans and failed phase II trials to assess its efficacy as a therapeutic vaccine (reviewed in Aurelian, 2004).

DNA Vaccines

Plasmid DNA encoding glycoproteins gD and/or gB or the IE protein ICP27 induced protective immunity in some animal models, but immunity was incomplete in other models. Expression of cell-associated forms of gD-2 induced primarily Th1 responses, whereas expression of secreted gD-2 resulted in a Th2 response. In guinea pigs, immunization with DNA encoding cytosolic gD-2 did not protect from acute or recurrent disease, whereas protection was seen in mice. The vaccines did not improve virus clearance from the inoculation site nor significantly reduce recurrent disease in guinea pigs when used for therapeutic vaccination. When present, protection was mediated by CD4$^+$ T cells. Coadministration of DNA encoding Th1 cytokines or chemokines increased the potency of these agents (reviewed in Aurelian, 2004). DNA vaccines do not seem to be clinically promising.

Therapeutic Vaccines

We developed a therapeutic vaccine that decreases the frequency of HSV-2 recurrences in phase II clinical trials. The vaccine, known as ICP10ΔPK, is a live HSV-2 mutant which has been rendered growth incompetent by deletion of ICP10PK. Under specific conditions, ICP10ΔPK can be grown in culture in the absence of complementing ICP10PK, thereby eliminating the risk of recombination associated with the other live-virus vaccine candidates. It also is deleted in the transforming sequences, increasing safety. ICP10ΔPK does not replicate nor cause disease in infected animals (mice and guinea pigs) and is defective in latency establishment, with only 12% of the ganglia from the vaccinated animals retaining viral DNA at 100-fold-lower levels than in ganglia from HSV-2-infected animals. ICP10ΔPK does not reactivate upon HSV-2 infection (Aurelian et al., 1999; Wachsman et al., 2001).

In mice, ICP10ΔPK provided virtually absolute protection from fatal and cutaneous HSV-2 disease. In guinea pigs, it reduced HSV-2 cutaneous lesions (6% with disease compared to 100% in the placebo group) and vaginal disease (10% with disease compared to 100% in the placebo group) and significantly reduced the ability of the challenge HSV-2 to establish latency (32% positive ganglia and 3×10^3 HSV-2 DNA/ganglion compared to 70% positive ganglia and 2×10^5 molecules/ganglion in vaccinated and unvaccinated animals, respectively). Protection extended to HSV-1 challenge (Aurelian et al., 1999; Wachsman et al., 2001). Significantly, ICP10ΔPK is the first vaccine with documented therapeutic activity. In guinea pigs previously infected with HSV-2, vaccination caused a 75 to 90% reduction in recurrent disease and reduced the frequency and duration of the recurrent episodes experienced by the rare animals that were not absolutely protected (Table 4). Protection was associated with the failure to induce IL-6 and/or IL-10 production in infected keratinocytes and elicitation of a Th1 response that included CD8$^+$ CTL through increased DC activation (IL-12 production) (Gyotoku et al., 2002). This immune profile is responsible for vaccine activity because HSV-2 replication and disease were inhibited by adoptive transfer of the CD8$^+$ CTL and/or CD4$^+$ Th1 cells (Gyotoku et al., 2002).

In phase I and II clinical trials, the ICP10ΔPK vaccine was tolerated well, and recurrences were completely eliminated in 44% of the vaccinated patients at a 1-year follow-up. This compares to 13% without recurrences in the placebo group ($P = 0.024$). The number of recurrent episodes was significantly reduced relative to the previous year ($P < 0.001$) and in the vaccinated group relative to the placebo group ($P = 0.04$) (Table 5). Efficacy was gender independent and was seen in both HSV-1- and HSV-2-seropositive subjects. Virus-specific CD8$^+$ CTL and CD4$^+$ Th1 cells mediated protection (Casanova et al., 2002; Aurelian, 2004). ICP10ΔPK is a critical development for patient management. By decreasing the frequency of recurrent disease and, thereby, transmission, ICP10ΔPK also promises to reduce disease prevalence. However, additional studies are needed to perfect the dosage necessary for further reduction of recurrent disease frequency, establish the maximal duration of protection, and define the impact of vaccination on asymptomatic virus shedding.

THERAPY

A critical advance has been the development of antiviral chemotherapy. Agents currently used for genital HSV are acyclovir, valacyclovir, and famciclovir (Table 6). Valacyclovir is the oral prodrug of acyclovir and has three- to fivefold-higher bioavailability than acyclovir. Famciclovir is the oral prodrug of penciclovir, the active antiviral compound. These drugs are nucleoside analogs structurally related to endogenous pyrimidine or purine bases. They are phosphorylated

TABLE 4 ICP10ΔPK has therapeutic HSV-2 vaccine activity in guinea pigs[a]

Recurrent cutaneous disease	Mock vaccinated	Vaccinated
No. of animals with lesions/total (%)	11/14 (79)	1/15 (7)
Day of 1st recurrence	21.9 ± 1.4	32
Duration (days)	10.4 ± 2.2	3
Peak lesion score	2.5 ± 0.2	2
Peak footpad width (mm)	0.56 ± 0.07	0.29
No. of recurrences/animal	2.4 ± 0.4	1

[a]Guinea pigs were infected with HSV-2 (3×10^6 PFU) and mock vaccinated (with phosphate-buffered saline) or vaccinated with ICP10ΔPK mutant (10^6 PFU) on days 7 and 17 after HSV-2 infection. Animals were monitored for 40 days for the development of recurrent HSV-2 cutaneous disease. Data are expressed as means ± standard errors of the means (Wachsman et al., 2001).

TABLE 5 ICP10ΔPK has therapeutic activity in phase II trials[a]

Recurrent disease	Result for group at:					
	6 mo			12 mo		
	Placebo	Vaccinated	*P*	Placebo	Vaccinated	*P*
No. of illness days/patient	18	10	0.028			
Mean no. of episodes/group	3.13	1.58	0.028	4.8	2.52	0.04
No. of episodes/mo (result for prior yr)	0.52 (0.84)[b]	0.26 (0.75)	<0.001			
% with no symptoms (<2 episodes)	0	37.5		13	43.5 (61)	<0.001

[a]Patients with frequent HSV-2 recurrences (>10/year) vaccinated with 10^5 PFU of ICP10ΔPK and monitored for development of recurrent disease for 6 and 12 months (adapted from Casanova et al., 2002).
[b]$P = 0.185$.

by HSV TK at a 10^6-fold-faster rate than the cellular TK. After additional phosphorylation by cellular enzymes, the triphosphate (active form) blocks viral DNA synthesis by acting as an inhibitor and substrate of viral polymerase, becoming incorporated into the growing DNA chain and causing termination of chain growth. HSV Pol has a much higher affinity for the drug than the cellular enzyme. The International Herpes Management Forum recommends acyclovir administered intravenously at 10 mg/kg of body weight every 8 h for 14 to 21 days as the treatment of choice for HSE. Even with early administration of therapy, nearly two-thirds of HSE survivors have significant residual neurologic deficits. Acyclovir may also be effective for the treatment of HSV-2-induced meningitis. For HSV-infected neonates the duration of parenteral acyclovir therapy is 14 to 21 days, dependent on the extent of disease. Use of subsequent oral suppressive therapy is under investigation (Kimberlin, 2007). However, as DNA synthesis is required for the drugs to function, they cannot destroy the virus during latency.

Episodic oral therapy speeds healing and resolution of primary and recurrent HSV lesions. Long-term daily suppressive (prophylactic) therapy may reduce the frequency of reactivation in patients with frequent recurrent episodes, but it does not eliminate ganglionic latency, and reactivation occurs after therapy is discontinued. Valacyclovir was reported to prevent recurrent HSV and decrease asymptomatic virus shedding when given as daily prophylaxis (Tyring et al., 2002; Baker and Eisen, 2003). Daily oral administration for 6 to 12 months appears to be safe, but a potential concern of long-term treatment is that nonreplicating HSV-2 is oncogenic. The efficacy of valacyclovir suppressive therapy in pregnant women with recurrent genital herpes is unclear. In one randomized and controlled study of women at 36 weeks of gestation, oral valacyclovir (500 mg) reduced the number with clinical HSV recurrences between the time of study onset and delivery (10.5% versus 27.3% in the placebo group; $P = 0.023$), but virus shedding within 7 days of delivery and the number of women with clinical HSV lesions at delivery were similar for both groups (Andrews et al., 2006). A mathematical model indicated that, at the current coverage rate of 3.2%, the incidence of HSV-2 would be only modestly reduced (1.8 to 2.8%) by suppressive therapy (Williams et al., 2007). Higher rates of diagnosis and a focus on coverage soon after infection may help increase its impact. Barrier prevention during intercourse and foreplay is recommended, and research is focused on the development of wide-spectrum microbicidal agents that can be applied vaginally to prevent infection (Kokuba et al., 1998).

Acyclovir resistance emerges with moderate frequency, and it crosses to valacyclovir and famciclovir. Foscarnet and cidofovir are used to treat infections caused by acyclovir-resistant virus. Foscarnet is a pyrophosphate analog. It inhibits viral DNA polymerase directly and does not need to be activated intracellularly. Foscarnet competes with pyrophosphate for the binding site on Pol. It has recently been developed for oral delivery (Pechere et al., 1998). Cidofovir undergoes 2 stages of phosphorylation via monophosphate kinase and pyruvate kinase, respectively, to form an active metabolite, cidofovir diphosphate, that bears structural similarity to nucleotides and acts as a competitive inhibitor and an alternative substrate for DNA polymerase. Nephrotoxicity is a potential complication with parenteral use of cidofovir, and it should be administered with oral probenecid after prehydration to minimize adverse effects. A topical cidofovir formulation is currently under investigation. Acyclovir-resistant HSV can become sensitive after treatment with cidofovir, suggesting that alternating acyclovir and cidofovir therapies may provide a strategy for managing the

TABLE 6 CDC treatment recommendations for genital HSV

Drug	Dose (mg) for[a]:		
	1st clinical episode (7–10 days)	Recurrent disease	
		Episodic (no. of days)	Suppressive (daily)
Acyclovir	400 t.i.d. 200 5 ×/day	400 t.i.d. (5) 800 b.i.d. (5) 200 5 ×/day (5)	400 b.i.d.
Valacyclovir	1,000 b.i.d.	500 b.i.d. (3–5) 1,000 q.i.d. (5)	500 q.i.d. 1,000 q.i.d.
Famciclovir	250 t.i.d.	125 b.i.d. (5)	250 b.i.d. (up to 1 yr)

[a]t.i.d., three times a day; b.i.d., twice a day; q.i.d., four times a day.

emergence of alternating acyclovir-resistant and -sensitive recurrences.

Ongoing research is focused on the development of additional agents to treat acyclovir-resistant strains. Presently developed compounds include a ribonucleotide reductase peptidomimetic inhibitor (BILD 1633) (Duan et al., 1998), a DNA polymerase inhibitor (viz., H2G) (Neyts et al., 1998), a class of nonnucleoside antiviral agents (viz., SCH 43478) that have selective activity against HSV-2 and function at the level of IE gene transactivation (Albin et al., 1997), and BAY57-1293, a novel nonnucleoside inhibitor of the HSV helicase-primase that suppressed clinical symptoms, reduced virus shedding, and diminished the frequency of recurrences in the guinea pig model (Baumeister et al., 2007). Antisense oligonucleotides that target IE genes also were reported to inhibit virus growth (Smith et al., 1986; Kulka et al., 1994; Aurelian, 1998) and could overcome drug resistance. However, improved delivery methods must be developed. Resiquimod, an investigational immune response modifier and TLR 7 and 8 agonist, stimulates production of cytokines that promote Th1 responses. In a randomized double-blind vehicle-controlled trial, resiquimod 0.01% gel (topical application 2 times weekly for 3 weeks) had a lower median lesion and shedding rate than vehicle control during the initial treatment, but recurrences occurred during the next 7 months (Mark et al., 2007a).

HSV VECTORS AND ONCOLYTIC VIRUSES FOR GENE THERAPY

Vectors for Gene Delivery to the CNS

In the CNS, apoptosis is triggered by a variety of stress conditions, including virus infection, ischemia, and excitotoxin release, and it has been associated with neurodegenerative diseases. Several strategies were proposed to interrupt the apoptotic cascade, including gene therapy with growth factors or antiapoptotic proteins delivered by virus vectors. The advantage of HSV-based vectors for therapeutic gene delivery to the CNS is that they are neurotropic. One type of HSV-1-based vector, known as amplicon, consists of a plasmid engineered to contain an HSV-1 origin of replication and packaging site along with a bacterial origin of replication in which a selected therapeutic gene is introduced. Amplicons are packaged by cotransfection with a defective HSV-1 helper virus, giving rise to a mixed population of virus particles that consist of the packaged amplicon and the helper virus, which is toxic. Safety was improved by the development of a helper-free delivery system that supports the replication and packaging of cotransfected amplicon DNA. However, safety concerns linger, as HSV-1 induces apoptosis in CNS neurons, causes severe and often fatal encephalitis in immunocompetent humans, and is associated with epileptiform seizures. HSV-1-induced apoptosis is through activation of the JNK/c-Jun pathway, which is also activated in the HSE brain (Perkins et al., 2003a; Perkins et al., 2003b) and is likely mediated by ICP0 (Fig. 7). Helper-free amplicon vectors did not demonstrate significant neuroprotection unless they were given at extremely high doses that were associated with toxicity, and they lost the HSV immune evasion properties, which interfere with repeated treatment, should it be required (Olschowka et al., 2003). Another type of HSV-1-based vector consists of virus mutants that were rendered growth compromised through deletion of IE genes. One such vector had a deletion of the IE gene ICP4, but it proved to be toxic and induced apoptosis. In the brain, transgene expression was transient, shutting off in a few days, even with neuron-specific promoters. Similar conclusions were reached for another HSV-1 mutant, which was had a deletion of the IE gene ICP27. Toxicity was eliminated by inactivation of all the IE genes, but these viruses are difficult to grow (no cell lines complement all IE genes) and transgene expression in the hippocampus was weaker than that seen for the ICP4 mutant (reviewed in Fink et al., 1996). Another HSV-1 mutant had a deletion of ICP4, ICP27, and $\gamma_1$34.5 and had a disabling mutation in VP16. Proof of principle experiments, using this mutant to deliver LacZ under the control of a promoter that contains elements of the LAT promoter, indicated that LacZ is expressed in organotypic hippocampal cultures for 23 days (Lilley et al., 2001). However, the design of all HSV-1 vectors suffers from limitations caused by (i) loss of innate antiapoptotic genes (viz., ICP27 and $\gamma_1$34.5) and (ii) retention of genes that activate the apoptotic JNK pathway (Perkins et al., 2003a).

HSV-2 is a superior gene delivery vector because it does not activate the JNK apoptotic pathway or cause encephalitis in adult humans, likely related to the neuroprotective activity of ICP10 PK (Perkins et al., 2003a; Perkins et al., 2003b). The HSV-2 mutant ΔRR is growth compromised and avirulent (even after intracerebral inoculation), and it delivers the viral antiapoptotic protein ICP10PK to the brain. It is neuroprotective in various paradigms of neuronal cell death in primary and organotypic hippocampal cultures and in animal models of excitotoxic neuronal cell death. Neuroprotection is through activation of redundant survival pathways, including Ras/Raf-1/MEK/ERK, PI3-K/Akt, and AC/PKA (Fig. 7). Most importantly, the surviving neurons retain functional (synaptic) activity, unlike other gene therapy platforms (Laing et al., 2008; Gober et al., 2006; Golembewski et al., 2007; Wales and Aurelian, 2007).

Oncolytic Viruses for Cancer Therapy

Oncolytic viruses are promising tools for cancer gene therapy. HSV has a number of characteristics that make it a useful oncolytic vector, including its large, sequenced genome that can accommodate multiple transgenes, its lack of insertional mutagenesis, its ability to infect a wide array of cell types in various species, and the availability of well-established antiviral therapies to treat it. The efficacy of oncolytic HSV therapy against schwannomas and malignant peripheral nerve sheath tumors has been studied in multiple experimental models both in vitro and in vivo. The virus utilizes cell pathways unique to tumors to enhance its oncolytic efficacy, preferentially and effectively targeting and destroying the tumor cells without harming normal cells. This effect is augmented by transgenes expressing antiangiogenic factors, such as dominant-negative fibroblast growth factor receptor and platelet factor 4, or Th1 cytokines (such as IL-12). Oncolytic viruses have synergistic activity with chemotherapy.

HSV-1 mutants G207 and MGH1 have double deletions in both copies of the neurovirulence gene $\gamma_1$34.5 and an insertional mutation in the R1 gene. They are avirulent upon intracerebral inoculation and were shown to kill CNS tumor cells (viz., glioma), decrease tumor growth, and prolong survival in tumor-bearing nude mice. Tumor specificity appears to be related to increased MEK activity in tumor, as compared to normal, cells. This function requires intact T-cell activity. Additional HSV-1-based oncolytic viruses were constructed to express IL-4, granulocyte monocyte colony-stimulating factor, or the CD40 ligand. An ICP10-PK-deleted HSV-2 mutant with an additional mutation in an as yet unidentified gene that imparts fusogenic activity,

and thereby increases virus replication (known as FusOn-H2), also kills various tumor cells in mouse xenografts (Fu et al., 2007). Others use the glioma-specific nestin promoter to drive deletion of $\gamma_1$34.5 (to increase tumor specificity) or of ICP47 (to reduce immune evasion) (reviewed in Shen and Nemunaitis, 2007). However, early clinical trials have shown that while oncolytic viral therapies are tolerated well, their efficacy is modest. This is related to poor virus replication and spread within the tumors, and it is becoming increasingly evident that the development of oncolytic viruses with distinct molecular death functions is highly desirable. Our recent studies indicate that the ΔPK virus has robust melanoma oncolytic activity in culture and in animal models (xenografts) through the simultaneous activation of multiple nonredundant programmed cell death pathways that include activation of distinct protease families and upregulation of the autophagy protein Beclin-1 and the pro-aptoptotic protein H11/HspB8 (Colunga et al., submitted for publication). Another cancer gene therapy approach (known as suicide) is based on the ability of HSV TK to preferentially activate ganciclovir, thereby killing tumor cells that contain the TK gene (delivered with an HSV-1, retrovirus, or adenovirus vector) as well as surrounding tumor cells (bystander effect) (reviewed in Jeyaretna et al., 2007).

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Cytomegalovirus, Varicella-Zoster Virus, and Epstein-Barr Virus

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CMV

History

Enlarged cells with intranuclear (IN) inclusion bodies were first noticed in 1904, which were later termed "cytomegalia" by Goodpasture and Talbot in 1921. Viral etiology, not protozoal as previously thought, was identified in 1926 after demonstration that the infectivity of the inclusion bodies was retained even after filtration. In 1956, the virus was independently isolated from human tissues in cell cultures by three groups (Rowe et al., 1956; Smith, 1956; Craig et al., 1957) and was referred to as the salivary gland virus or cytomegalic inclusion disease virus. The term cytomegalovirus (CMV) was proposed by Weller and colleagues, based on its antigenic diversity (Weller et al., 1960; Weller, 2000). Current taxonomy refers to the virus as human herpesvirus type 5.

As the initial studies were focused on congenital CMV infections in infants, it was considered a cause of disease in children only. However, its disease spectrum was broadened when it was shown to cause infectious mononucleosis (IM)-like illness in immunocompetent adults (Klemola et al., 1965) and more serious disease in immunosuppressed organ transplant recipients (Hill et al., 1964; Ho, 1977).

Biology

Characteristics

To distinguish it from the CMVs in other animals, human CMV is often abbreviated HCMV. In this chapter, however, unless otherwise specified, CMV refers only to human CMV. CMV is a member of the *Betaherpesvirinae* subfamily of the *Herpesviridae* family and, as such, shares the typical structure of a herpesvirus. The viral nucleocapsid is icosahedral in symmetry, about 150 to 200 nm in diameter, and is comprised of 162 hexagonal capsomeres. This is surrounded by an amorphous tegument and lipid envelope. The tegument is comprised of mostly phosphoproteins, while the outer envelope consists of lipoproteins and structural proteins, some of which are glycosylated. Glycoproteins such as gB and gH proteins share genetic homology with herpes simplex virus (HSV) envelope proteins, from which their commonly used names are derived. Similar to other enveloped viruses, CMV is readily inactivated by heat, organic solvents, and dessication.

Viral Genome

The CMV genome is the largest among all human herpesviruses and is divided into the unique short (US) and unique long (UL) regions, both of which are bound by repeat sequences (RL and RS). Both regions can be inverted, giving CMV the ability to form four isomers, all of which are produced in equal amounts in culture. The genomic size of clinical isolates (Merlin, a low-passaged wild-type CMV strain; 235,645 bp) is larger than that of the laboratory strain AD169 (230,283 bp), which has a deletion of at least 19 genes in the UL (UL133 to UL150) region and frameshift mutations in 3 genes (RL5A, RL13, and UL131A) (Prichard et al., 2001; Akter et al., 2003; Davison et al., 2003; Dolan et al., 2004). Genetic variabilities within CMV strains can affect host cell tropism, replication efficiency, and pathogenicity. Clinical isolates with an intact UL128 to UL150 gene segment can efficiently infect endothelial and epithelial cells (Ryckman et al., 2006; Wang et al., 2005c). Of the seven genomic variants of the gN (gN-1, gN-2, gN-3a, gN-3b, gN-4a, gN-4b, gN-4c) gene, genotype gN-1 was found to be highly prevalent (87%) during latent infection in a healthy immunocompetent population and less prevalent (24%) in solid-organ transplant recipients with CMV disease and symptomatic congenital CMV infections (19%) (Pignatelli et al., 2003; Rossini et al., 2005). None of the gB genotypes was associated with disease progression and clinical outcomes during CMV infections in immunocompromised transplant recipients and congenital CMV disease (Humar et al., 2003; Picone et al., 2004a).

Recent studies identified multiple microRNAs (miRNA) encoded by CMV during infection, possibly during early stages of replication (Grey et al., 2005; Pfeffer et al., 2005). These miRNAs function as small interfering RNAs and may be able to regulate cellular or viral gene functions during infection.

Viral Proteins

CMV has approximately 200 to 250 open reading frames (ORFs) with protein coding capacity (Murphy et al., 2003a; Murphy et al., 2003b), although the functions of most of these proteins have not yet been identified. Quantitative analysis of viral proteins shows that the virion comprises 50% tegument proteins, 30% capsid proteins, 13% envelope

proteins, and 7% undefined proteins. Overall the most abundant protein in the virion is the pp65 (UL83) tegument protein.

Compared to other herpesviruses, the CMV genome encodes the largest number of glycoproteins. Fifty-seven glycoproteins are encoded by the AD169 strain, and 72 glycoproteins are encoded by clinical isolates with a wide variety of functions (Pignatelli et al., 2004). These include glycoproteins gB (gpUL55), gM (UL100), gH (UL75), gL (UL115), gO (UL74), and gN (UL73). A recent study has shown that gM (10%), not gB (1%), is the most abundant protein in the CMV envelope (Varnum et al., 2004). Some of these glycoproteins exist as complexes such as gc-I (gB), gc-II (gM and gN), and gc-III (gH, gL, and gO). Neutralizing antibodies against gB, gH, and gM/gN have been identified in infected individuals. The coiled coil domains of glycoproteins gB and gH are involved in fusion of the virus to the host cell. gM and gN are the major heparin binding factors and may be involved in viral attachment (Pignatelli et al., 2004). gH and gL play a role in intracellular transport of the virion. Tegument proteins such as ppUL32, pp150, and pp28 are expressed in the cytoplasm, ppUL69 is expressed in the nucleus, and ppUL53 and ppUL83 (pp65) are expressed in the nucleus during early infection and then translocated to the cytoplasm during late infection (Sanchez et al., 2000). Several tegument proteins are involved in viral gene expression (pp71), replication (pp28), nuclear egression of pro-capsids (ppUL31), and modifying host cellular responses such as cell cycle arrest (ppUL69) (Britt and Boppana, 2004). Capsid proteins such as pUL86, pUL48-49, pUL85, pUL46, and pUL80 form the pentons, hexons, and the triplexes in the icosahedral structure of viral capsid (Britt and Boppana, 2004). Core proteins such as pUL54, pUL44, pUL57, pUL105, pUL70, and pUL102 are involved in viral DNA replication.

Cell Tropism

CMV can replicate in vitro in fibroblasts, macrophages, endothelial cells, epithelial cells, trophoblasts, hepatocytes, and oral mucosal cells (Gilloteaux and Nassiri, 2000; Sindre et al., 2000; Fisher et al., 2000; Wang and Shenk, 2005; Ryckman et al., 2006; Hai et al., 2006). In vivo, CMV can infect epithelial cells of the kidney, liver, bile ducts, salivary gland, gut epithelium, lung, and pancreas as well as endothelial cells. Furthermore, CMV infection has been described in polymorphonuclear leukocytes (PMNs), monocytes, monocyte-derived dendritic cells (DCs), and T and B lymphocytes (Razonable et al., 2002; Gerna et al., 2004). Although clinical isolates of CMV have been derived from a wide variety of cell types, the laboratory-adapted strains such as AD169 and Towne have very low efficiency to infect endothelial and epithelial cells. The molecular basis for this cell tropism is linked to the expression of UL128 to UL150 ORFs (Wang et al., 2005c; Ryckman et al., 2006). Clinical isolates of CMV with wild-type UL128 to UL150 ORFs can efficiently infect both epithelial and endothelial cells, whereas these ORFs are either deficient or mutated in the laboratory-adapted strains. Furthermore, in addition to endothelial cell tropism, the UL131 to UL128 gene locus is also shown to be involved in virus transfer to the leukocytes (Hahn et al., 2004). Similarly, deletion of the UL132 ORF was shown to reduce the efficiency of CMV replication in fibroblasts. Cellular differentiation is a major factor involved in reactivation of latent CMV infection (Soderberg-Naucler et al., 2001). Longer-lived $CD34^+/CD38^-$ bone marrow progenitors, $CD14^+$ monocytes, and endothelial cells are important in latent and acute CMV infections (Jarvis and Nelson, 2002; Goodrum et al., 2004). Epithelial cells within organs are a major target of CMV infection. Despite its broad host range for cell attachment, human CMV replication is extremely species specific, both in vivo and in vitro. While baboon CMV is transmitted to humans after baboon-to-human xenotransplantation (Michaels et al., 2001), the replication of human CMV in primate fibroblasts has not been demonstrated.

Replication Pathway

CMV exhibits the typical herpesvirus replication cycle with initial attachment to cell membrane receptors. Indeed, heparan sulfate proteoglycans and integrins have been implied to have a role in viral entry (Liu and Thorp, 2002; Feire et al., 2004). DC-specific ICAM-grabbing nonintegrin binds gB and facilitates viral entry into the cells (Halary et al., 2002). Epidermal growth factor receptor was shown to bind gB (Wang et al., 2003b) along with cellular integrin $\alpha v\beta 3$ as a coreceptor (Wang et al., 2005c). However, more recently it was shown that epidermal growth factor receptor was not required for CMV entry into cells (Isaacson et al., 2007).

The coiled coil domains of glycoproteins gB and gH are involved in fusion of the virus to the host cell. Following fusion of the viral envelope with the cell membrane, the nucleocapsid of CMV enters the cell and travels to the nucleus. Some of the tegument phosphoproteins also are translocated to the nucleus while others remain in the cytoplasm. The viral DNA moves from the nucleocapsid into the nucleus and an alpha-beta-gamma sequence of gene expression follows. The immediate-early (IE) or alpha gene proteins, primarily transactivators, appear in the infected cell first, followed by the early or beta gene proteins, primarily nonstructural glycoproteins. The major IE promoter (MIEP) of CMV controls the transcription of IE genes, IE1 and IE2, to encode proteins p72 and p86, respectively. Thus, MIEP is crucial in initiating the activation or reactivation of replication. MIEP is in turn regulated by cellular factors such as activation of signaling through NF-κB and induction of proinflammatory cytokines, cellular differentiation, and the Daxx protein (DeMeritt et al., 2004; Cantrell and Bresnahan, 2006; Yee et al., 2007). In addition to cellular factors, viral chemokine receptor gene homolog US28 is also involved in stimulating MIEP activity (Boomker et al., 2006). Tegument proteins pp71 (UL82) and ppUL35 interact with each other to activate MIEP (Schierling et al., 2004). The expression of IE2 gene is necessary for the subsequent activation of early gene transcription and DNA replication (Marchini et al., 2001).

The early proteins (beta proteins) are required for initiation of DNA replication. Rolling circle replication of the viral DNA occurs next and requires at least 11 virally coded proteins that include six core proteins and five noncore proteins pUL84, IE2, and pUL36-38. UL84 interacts with IE2 to activate oriLyt-dependent DNA replication (Colletti et al., 2004; Xu et al., 2004a; Xu et al., 2004b). Replication is initiated at the *cis*-acting origin of replication, oriLyt, near the center of the U_L region of the genome (Borst and Messerle, 2005). During this time, the production of late or gamma gene proteins, primarily structural components of the progeny virions, also takes place. Appropriate packaging of DNA is achieved in the nucleus by a "headfull" mechanism, with cleavage of concatemers occurring when no more DNA can be inserted into the capsid. These capsids then undergo nuclear egression to enter the cytoplasm, and the conserved UL31 and UL34 genes are possibly involved in

this process (Mettenleiter, 2002; Mettenleiter et al., 2006). The capsids obtain final tegument and envelope glycoproteins in the cytoplasm and during budding into the vesicles of the *trans*-Golgi compartment. The mature viral particle is then transported to the plasma membrane in a cellular vesicle, where the vesicle fuses with plasma membrane and the virus is released from the cell. Tegument protein pp150 (ppUL32) is required for the final egression of the virion from the cell, and its deletion resulted in accumulation of viral DNA in the cytoplasm with absence of cell-to-cell spread (AuCoin et al., 2006). Propagation of CMV in appropriate cell cultures produces progeny viral particles, dense bodies, and noninfectious enveloped particles. Despite its reputation as a relatively slow-growing virus in vitro, CMV is far more dynamic in vivo with an average doubling time or half-life in peripheral blood of approximately 1 day (Emery, 1999).

Epidemiology and Clinical Manifestations

CMV has a ubiquitous worldwide distribution and is nonseasonal. Seroprevalences range from 30 to 100% depending on age, geographic location, and ethnic and socioeconomic background. In general, the seroprevalence in resource-poor countries is close to 100%, and infections tend to be acquired early in life (Kothari et al., 2002). In the United States, the seroprevalence of CMV is around 40 to 50% (Staras et al., 2006). Populations in developed countries tend to exhibit lower seroprevalence rates, and infections are more commonly acquired during adolescence and adulthood, with higher rates of acquisition in lower socioeconomic populations. The prevalence of congenital CMV infection in the United States is 0.5 to 1.5% and about 10 to 15% of congenital CMV infections are symptomatic at birth (Ross et al., 2006a). About 20 to 30% of preschool children are infected with CMV.

Clinical Manifestations

The host immune response is critical in determining the severity of illness caused by CMV infection. Primary infection is usually self-limited in postperinatal immunocompetent hosts and is either asymptomatic or may present with mononucleosis-like illness with fever and malaise, myalgias, lymphadenitis, and mild hepatitis for a few weeks. Rare complications are pneumonitis, myocarditis aseptic meningitis, and Guillain-Barre syndrome (Sissons and Carmichael, 2002). The virus persists in the immunocompetent host in a latent stage until reactivated by an immunocompromising condition. More serious manifestations of CMV disease are seen in immunocompromised hosts and in congenital and perinatal CMV infections.

Congenital and Neonatal CMV Infection

CMV infection is one of the commonest of the TORCH (toxoplasma, rubella, CMV, and herpes simplex virus type 1 [HSV-1]) group of organisms causing birth defects, with an incidence of 0.15 to 2.0% in developed countries (Stegmann and Carey, 2002; Gaytant et al., 2003). It is acquired by maternofetal transmission of CMV via the hematogenous route across the placenta during pregnancy. Though still controversial, polymorphisms in the UL144 gene have been associated with congenital CMV infections (Arav-Boger et al., 2002; Picone et al., 2004b). Although congenital CMV disease can occur in pregnant women with primary or recurrent CMV infection, the risk of acquiring symptomatic disease in the newborn is higher (30 to 40%) after primary maternal infection than in recurrent infection (1 to 2%) (Fowler et al., 2003; Ross et al., 2006a). Symptomatic congenital CMV disease manifests as microcephaly, intrauterine growth retardation, deafness, hepatosplenomegaly, chorioretinitis, thrombocytopenia, hepatitis, and pneumonitis. Congenital CMV infection asymptomatic at birth may lead to hearing loss in later years (Ross et al., 2006b). Perinatal CMV infection can also be acquired by babies exposed to CMV infection in the maternal genital tract during birth or in nurseries after birth as well as through breast milk feeding from a CMV-infected mother. Perinatal CMV infections are usually mild and self-limiting in full-term babies. Laboratory diagnosis of congenital CMV disease should be made early within the first couple of weeks after birth, as it is not possible to differentiate it from perinatal CMV infection in older babies.

Immunocompromised Hosts

HIV-1–AIDS. Opportunistic CMV infection in AIDS patients predominantly presents as CMV retinitis, which is rarely seen during typical CMV disease in organ transplant recipients. Other sites of CMV end organ disease are the gastrointestinal tract and the central nervous system (CNS). AIDS patients with $CD4^+$ T cell counts of less than 50 to 100 cells/mm^3 have a higher risk of developing end organ CMV disease (Salmon-Ceron et al., 2000). Another risk factor determining CMV disease progression is high CMV viral load in these patients (Spector et al., 1999; Deayton et al., 2004). While still controversial, CMV is considered to act as a cofactor for human immunodeficiency virus type 1 (HIV-1)–AIDS infection (Robain et al., 2000; Robain et al., 2001; Griffiths, 2006). However, a significant decline in serious manifestations of CMV disease in AIDS patients has occurred since the introduction of highly active antiretroviral therapy (HAART) (Drew, 2003; Goldberg et al., 2005). A side effect of HAART during CMV infection in AIDS patients is immune reconstitution syndrome, which commonly manifests as vitritis and is caused by increased CMV-specific inflammatory immune responses in the eye. This condition resolves completely upon treatment with steroids (Mutimer et al., 2002; Stone et al., 2002).

Organ transplant recipients. Significant morbidity and mortality are seen in CMV infections in immunosuppressed patient solid-organ and cell transplant recipients (Razonable, 2005; Razonable et al., 2005). These may be the result of either primary infection, reactivation of latent virus, or superinfection with a different strain. Symptoms may present as a variety of syndromes or disease states ranging from mild fever to multiorgan damage. The risk factors for CMV disease in organ transplant recipients are the CMV serostatus of the organ donor and recipient, use of antithymocyte antibodies such as OKT3 for immunosuppression, high CMV levels, and graft-versus-host disease. The recipient is at high risk for CMV disease if he or she is CMV seronegative and the donor is seropositive. In bone marrow transplant recipients, the high risk exists even if the recipient is previously seropositive. The clinical onset of CMV disease usually starts within 100 days of transplantation. The usual clinical findings are fever, leukopenia, thrombocytopenia, hepatitis, and rarely, pneumonitis or pancreatitis (Rowshani et al., 2005). Atypical findings such as absence of fever, leukocytosis, and absence of thrombocytopenia in late onset (>6 months posttransplant) CMV disease also have been reported (Slifkin et al., 2001). Invasive CMV infection can also be found histologically in the transplant organs. These clinical

findings are due to the direct infectious effects of viral replication. Indirect effects due to immune and inflammatory responses against CMV can frequently cause acute or chronic graft rejection (Cainelli and Vento, 2002).

Pathogenesis

Viral Transmission

Following a primary infection, the virus may be shed for months or, particularly in the case of perinatal infections, years in numerous body fluids including blood, urine, oropharyngeal secretions, breast milk, cervicovaginal secretions, and semen. CMV is also sexually transmissible, and this route of transmission is associated with primary CMV infection in pregnant women (Numazaki et al., 2000). Iatrogenic transmission of the virus can occur via blood transfusion and during solid-organ or bone marrow transplantation procedures (Castagnola et al., 2004; Kothari et al., 2002; Roback et al., 2006; McDevitt, 2006; Fishman et al., 2007). Reactivation of latent CMV infection following immunosuppressive drug therapy in organ transplant recipients can lead to more severe CMV disease.

Immune Response

Humoral Immune Response

Various major antigenic determinants induce CMV-specific humoral immune responses such as gB, gH, gM/gN, and ppUL25 (Alberola et al., 2000; Lazzarotto et al., 2001a; Furebring et al., 2002; Shimamura et al., 2006). The neutralizing antibody response against gB is found to be high during primary infection (Alberola et al., 2000). Antibodies against gM/gN can also be detected following natural infection (Shimamura et al., 2006). The protective role of the CMV-specific humoral immune response is controversial but is supported by the fact that the severity of illness is lower in CMV-seropositive immunosuppressed organ transplant recipients than primary CMV infection in seronegative recipients. Furthermore, passive CMV immunoglobulin is commonly used for prevention or treatment of CMV disease following organ transplantation. Detection of CMV-specific antibodies is commonly used for serological diagnosis of primary infection as described in a later section of this chapter.

Cellular Immune Response

Cellular immunity plays a major role in protection against progression of severe CMV disease. Components of the innate immune system such as natural killer (NK) cells, alpha/beta interferon (IFN-α/β), mannose-binding lectins, and the Toll-like receptors (TLRs) are reported to be important in conferring resistance to CMV infection (Webb et al., 2002, Iversen et al., 2005; Sainz et al., 2005; Boehme et al., 2006; Manuel et al., 2007). Pattern recognition receptors such as TLR2 and CD14 recognize viral glycoproteins gB and gH and initiate inflammatory cytokine responses (Compton et al., 2003; Boehme et al., 2006). Liver transplant recipients with a TLR2 polymorphism have higher viral levels than those with wild-type TLR2, suggesting its role in immune control of CMV replication (Kijpittayarit et al., 2007). NK cells are important in controlling early CMV replication and can exhibit antiviral cytotoxicity without prior sensitization. The mechanism of this resistance is proposed to be via interaction between NK cell activation receptor Ly49H and a virally encoded molecule that is upregulated on CMV-infected host cells (Brown et al., 2001; Webb et al., 2002). Antiviral effects of IFN-α/β and IFN-γ are another important defense mechanism of the innate immune system. IFN-α/β is induced in response to viral infections possibly via the TLR signaling pathway, while IFN-γ is produced by activated NK cells and T cells. Both IFN-α/β (type I) and IFN-γ (type II) synergistically reduce CMV replication, possibly by regulating IE gene expression (Sainz et al., 2005).

The principal components of cell-mediated adaptive immunity are the $CD4^+$ and $CD8^+$ T cells. CMV-specific $CD4^+$ and $CD8^+$ T cell responses are found in seropositive healthy individuals (Dunn et al., 2002; Sinclair et al., 2004). These responses were found to be directed against 151 CMV ORFs and utilize about 10% of peripheral blood $CD4^+$ and $CD8^+$ memory T cells (Sylwester et al., 2005) during asymptomatic latent CMV infection. Although major histocompatibility complex (MHC) class I restricted $CD8^+$ cytotoxic T lymphocyte (CTL) responses are predominantly focused against pp65 and IE-1 antigens, a broader repertoire of CMV antigens that induced strong CTL responses has been recognized (Elkington et al., 2003). Strong $CD8^+$ CTL responses are targeted against pp65, pp50, IE-1, gB, and IE-2, while weak responses are directed against pp28, pp150, pp71, US2, US3, UD6, US11, US16, and US18 proteins (Gandhi and Khanna, 2004). The presence of CMV-specific cytotoxic $CD8^+$ T cells correlates with recovery from acute CMV infection and prevention of disease in transplant recipients (Gratama et al., 2001; Bunde et al., 2005). $CD4^+$ T cells also are important in CMV host defense and have been studied during primary and latent CMV infection (Rentenaar et al., 2000; Gamadia et al., 2003; van Leeuwen et al., 2006). Evidence for the protective role of $CD4^+$ T cells was reported by Sester and colleagues who observed that a decrease in $CD4^+$ T cell counts correlated with onset of CMV disease in renal transplant recipients (Sester et al., 2001). Furthermore, adoptive transfer of CMV-specific polyclonal T cell lines containing both $CD4^+$ and $CD8^+$ T cells reduced the levels of virus in antiviral drug-resistant patients (Einsele et al., 2002). During acute CMV infection, $CD4^+$ T cells are proliferating and secrete IFN-γ (Rentenaar et al., 2000; Gamadia et al., 2003). After the acute infection subsides, the $CD4^+$ T cells return to a resting stage with reduction in IFN-γ-producing cells. Kinetic studies of $CD4^+$ T cells show that during asymptomatic infection, CMV-specific $CD4^+$ T cells peaked immediately after viral DNA is detected, which is followed by the appearance of CMV-specific antibodies and, lastly, by $CD8^+$ T cells. During symptomatic CMV infection however, the appearance of $CD4^+$ T cells was delayed and peaked after the appearance of CMV-specific antibodies and $CD8^+$ T cells (Gamadia et al., 2003). This suggests that the $CD8^+$ T cells alone are not sufficient to control viral replication and require help from effector $CD4^+$ T cells. In addition to CMV-specific $CD8^+$ CTL responses, HLA class II-dependent cytotoxic $CD4^+$ T cell responses directed against CMV-infected cells also have been described (Lindsley et al., 1986; Tazume et al., 2004; van Leeuwen et al., 2006; Casazza et al., 2006). Similar to $CD8^+$ CTL, these $CD4^+$ CTL responses are involved in clearance of CMV infection by exerting direct cytolytic activity against CMV-infected cells and by inhibiting viral replication via production of IFN-γ and tumor necrosis factor alpha (Hengel et al., 1994; Tazume et al., 2004; Casazza et al., 2006). During coinfection with HIV-1, CMV-specific $CD8^+$ T cells are increased in numbers compared with that during CMV infection in HIV-uninfected healthy individuals. These $CD8^+$ T cells produce IFN-γ and perforin targeting CMV-specific antigens, thus playing a role in preventing

disease progression during HIV-1 infection (Bronke et al., 2005; Stone et al., 2005; Stone et al., 2006).

Mechanisms of Latency

Infection with CMV results in lifelong latency of the virus with possible reactivations at various times caused by a variety of triggers. During latency, the viral genome persists intracellularly without undergoing replication to produce infectious virions, and the host remains asymptomatic until reactivation. CMV latency-associated transcripts are mapped to the IE1/IE2 region. Antibodies to proteins that may be expressed by latency-associated transcripts were detected in healthy individuals (Landini et al., 2000). Expression of IE genes is critical for further downstream expression of early and late genes and initiating productive infection. During latency, the IE genes are not expressed and the virus persists at low levels. The virus uses various strategies to escape detection by the host immunosurveillance mechanisms and maintain long-term persistence. Latent CMV infection is speculated to be cell type dependent, with the principal reservoirs being $CD34^+$ stem cells, $CD14^+$ monocytes, and endothelial cells (Jarvis and Nelson, 2002; Goodrum et al., 2004). Viral IE gene expression is halted so that viral antigens are not detected by the host's immune system. CMV infection inhibits cell cycle progression (Kalejta and Shenk, 2002) and blocks cellular apoptosis (Skaletskaya et al., 2001) to enhance its survival in infected cells. Reactivation of latent CMV infection is associated with cellular differentiation (Soderberg-Naucler et al., 2001), and the longer-lived primitive $CD34^+/CD38^-$ progenitor cells of the myeloid lineage support latent CMV genomes that could be reactivated into productive infection (Goodrum et al., 2004). Inflammatory cytokines such as IFN-γ can stimulate $CD14^+$ monocytes to differentiate into MDM that are permissive to productive infection (Soderberg-Naucler et al., 2001).

Immunosuppression and Immune Evasion

CMV overcomes or escapes detection by the host surveillance system by various mechanisms. Depressed $CD4^+$ T cell responses to mitogens and antigens persist for many weeks during CMV mononucleosis in otherwise immunocompetent adults (Rinaldo et al., 1977; Rinaldo et al., 1980; Levin et al. 1979). This could be related to lower MHC class II expression on antigen-presenting cells that is crucial for inducing $CD4^+$ T cell immune responses (Hegde et al., 2002). Protein products from several genes in the US region of CMV downregulate MHC class II expression. More profound effects, however, are due to genes within the US2 to US11 region that code for proteins that modulate MHC class I, as mutated strains with deletion of this region fail to downregulate MHC class I (Mocarski, 2004; Khan et al., 2005). US3 is expressed as an IE protein, US2 and US11 are expressed as early proteins, and US6 is a late protein, thus affecting MHC class I downregulation at different stages of viral replication (Mocarski, 2004). These genes have been shown to target different stages of the MHC class I antigen presentation pathway, e.g., preventing translocation of MHC class I to the cell surface (Basta and Bennink, 2003) and targeting proteosomal degradation of MHC class I molecules (Barel et al., 2006). US2 and US3 genes can also inhibit MHC class II antigen presentation pathway by either binding to or degrading MHC class II molecules (Hegde et al., 2002). Additionally, CMV encodes a viral interleukin-10 homolog (Kotenko et al., 2000), which, similar to its human counterpart, reduces the cell surface expression of MHC class I and class II proteins (Spencer et al., 2002). The IE genes reduced the expression of Janus kinase 1 (Jak-1) protein, thus downregulating IFN-γ-inducible MHC class II upregulation (Miller et al., 2002).

CMV is also shown to impair antigen presentation by DCs by inhibiting their maturation (Beck et al., 2003). CMV also codes for certain G-protein-coupled protein receptor and cytokine homologs. Four G-protein-coupled protein receptor homologs, US27, US28, UL33, and UL78, have been identified, of which US28 has been studied the most. US28 encodes homologs for most CC chemokines and the CX3C chemokine fractalkine receptors (Rosenkilde et al., 2001). These US28 chemokine receptor homologs can bind beta chemokines such as RANTES/CCL5, MCP-1/CCL2, and MIP-1α/CCL3, an event that is followed by a rise in intracellular calcium and intense sequestering of extracellular chemokines, thus reducing the inflammatory environment (Boomker et al., 2005). The US28-infected cells can also migrate towards the site of inflammation, facilitating viral dissemination (Hengel and Weber, 2000).

Diagnosis

Laboratory-based diagnosis is usually required to identify congenital and perinatal CMV disease and to diagnose and monitor viral levels in immunosuppressed hosts. The laboratory techniques commonly employed are the conventional tube and shell vial viral culture techniques, immunological techniques for histopathology, immunohistochemical techniques, antigen and antibody detection, and nucleic acid detection.

Histopathology

One hundred years after histopathology was first used to identify CMV infection, this method is still useful in recognizing the formation of typical "owl's eye" IN inclusion bodies in enlarged infected cells. These can be observed in numerous tissues, including kidney, liver, lung, and gastrointestinal tract, in persons with systemic CMV infection. Confirmation of CMV by histological assessment of biopsy or autopsy tissue is done by the use of immunohistological procedures (Mattes et al., 2000; Dimitroulia et al., 2006; Lautenschlager et al., 2006). Cytological assessment of cells such as those from bronchoalveolar (BAL) specimens is used to monitor CMV infection in lung and heart transplant recipients (Tikkanen et al., 2001; Chemaly et al., 2004). In situ PCR and hybridization on tissue sections is shown to have lower sensitivity than PCR using other specimen types.

Viral Culture

CMV is usually isolated for diagnostic purposes by culture in human fibroblasts. However, the appearance of CMV cytopathic effect (CPE), while typically requiring 1 to 2 weeks of incubation, can take as long as a month. The shell vial assay is a modification of conventional viral culture in which centrifugation-amplified culture is combined with IE antigen (p72) detection (Galiano et al., 2001; Keightley et al., 2006). This method provides results within 24 to 48 h of inoculation and can be applied to a variety of specimens. Diagnosis of congenital CMV disease has classically been made by the isolation of CMV from urine or saliva within 1 week of birth, since these babies excrete very high titers of virus that rapidly cause CPE. Isolation after this time will not differentiate congenital from perinatal infection.

Serology

CMV-specific immunoglobulin M (IgM) antibodies appear within 2 to 4 weeks postinfection, while IgG antibodies

appear after 4 weeks. Enzyme immunoassays are usually employed to detect anti-CMV antibodies. Detection of CMV IgG antibody is used for diagnosis of CMV infection and not disease, as a majority of healthy individuals are seropositive for CMV in many geographic regions. Detection of IgM antibodies is not a reliable indicator of primary infection, as it may persist longer in some individuals or may not be produced in some transplant recipients (Halling et al., 2001; Mace et al., 2004). Despite these limitations, CMV serology is widely used for identification of primary CMV infection in pregnant women and in organ donors and recipients who have been infected with CMV. Measurement of the CMV-specific IgG avidity index is used to complement IgM detection. IgG avidity antibody production matures after the primary infection from low to high levels during several months and is maintained at high avidity for several years. Therefore, detection of a low-avidity IgG antibody index indicates primary infection, while detection of a high-avidity index rules out primary infection (Lazzarotto et al., 2001b; Mace et al., 2004).

Antigen Detection

Direct antigen detection for CMV diagnosis has been applied primarily to specimens of peripheral blood, cerebrospinal fluid (CSF), and BAL fluid. Direct detection of early (p72) and late (pp65) antigens with pooled antibody reagents can be used to detect CMV in the cellular component of BAL specimens. Quantitation of pp65 antigen by immunofluorescence staining in peripheral blood PMNs has higher sensitivity than viral culture and is commonly used to detect and monitor CMV viral load in organ transplant recipients (St. George et al., 2000). It is also used to initiate preemptive antiviral treatment at appropriate cutoffs, which may vary depending upon the patient population. A recent study reported a significant association between pp65 antigenemia and gN genotypes, suggesting its role in predicting disease severity. gN-1 was associated with low antigenemia and gN-4b with high antigenemia values, thus showing differences in virulence between the two genotypes (Rossini et al., 2005).

In comparing the CMV antigenemia assay to other types of diagnostic tests, it is important to understand its biological basis. The antigenemia assay is a manual, microscopic enumeration of the number of immunofluorescent PMNs expressing pp65. However, it is an indirect measure of CMV replication in the host, as PMNs do not support productive CMV replication (Rinaldo et al., 1977). Rather, detection of this viral matrix phosphoprotein in the PMNs is primarily due to uptake of other types of dying cells that support productive, lytic CMV infection or CMV virions and pp65 that are made in abundance in productively infected cells (Grundy et al., 1998; Gerna et al., 2000; Kas-Deelen et al., 2001). After internalization by the PMNs, the signal sequence in pp65 results in trafficking of the viral phosphoprotein to the nucleus (Hensel et al., 1996), where it can be detected by anti-pp65 monoclonal antibodies in the antigenemia assay.

The CMV antigenemia test has several limitations, including its requirement of more blood than molecular-based assays, it is very labor intensive, it does not lend itself to automation, it must be performed on blood soon after collection, and it can be subjective in its quantitation and interpretation. Utility of the assay is also hampered by specimens with low white cell counts, particularly in bone marrow transplant recipients or in blood that has degraded during transit to the laboratory, e.g., older than 24 h or due to extremes in temperature.

Nucleic Acid Detection

Molecular techniques employed for diagnosis of CMV are PCR, DNA hybridization assays (e.g., Digene Hybrid Capture CMV DNA assay) for CMV DNA, and nucleic acid sequence-based amplification for CMV RNA detection. Compared with DNA hybridization assays, PCR assays show higher sensitivity and specificity and faster turnaround time (Hanson et al., 2007). Recent years have seen advancement in PCR technology with the introduction of real-time PCR (Herrmann et al., 2004; Ikewaki et al., 2005), which is more sensitive, specific, and rapid than conventional PCR and allows quantitation and closed-tube detection. The real-time PCR platform uses fluorescence-based detection of PCR products that allows monitoring of amplicons in "real-time mode." Intercalating fluorescent dyes (e.g., SYBR green) or target-specific nucleotide probes labeled with reporter dyes are used to generate a fluorescence signal during each amplification cycle. Three types of probes, namely hydrolysis (TaqMan), hybridization, and molecular beacons, which rely on fluorescence resonance energy transfer between two adjacent dyes, are routinely used in clinical diagnostics. In the 5′ nuclease hydrolysis (TaqMan) probe and molecular beacon, the two dyes (reporter and quencher) are attached to a single probe, whereas the hybridization probe requires two probes labeled with one dye molecule. Multiplex real-time PCR assays using target-specific probes labeled with distinct reporter dyes have been described for simultaneous detection of multiple herpesviruses in a single PCR tube (Wada et al., 2007).

CMV DNA can be detected in different specimens, such as blood, BAL fluid, CSF, and tissue specimens, depending on the site of infection. Although different components of blood, such as whole blood, plasma, peripheral blood leukocytes, and peripheral blood mononuclear cells, are used to analyze CMV DNA levels, use of whole blood for CMV PCR is recommended (Razonable and Emery, 2004). Indeed, CMV viral load is 0.67 $\log_{10}$ higher in whole blood than in plasma (Razonable et al., 2002). Several commercial and in-house real-time PCR tests are available for monitoring CMV DNA levels in blood. However, since there is no standardization regarding use of specimen types, target gene, calibrators, detection platforms, and nucleic acid extraction methods, comparisons of CMV PCR results among different laboratories are difficult. Genetic differences between PCR target regions can give variable CMV copy numbers. Discrepant CMV DNA copy numbers for the polymerase (*pol*) and glycoprotein B (*gB*) genes have been noted in 2 patients based on 4 nucleotide mismatches in the *gB* fragment of the primers (Herrmann et al., 2004). Another study found US17 to be more sensitive than UL65, *IE* protein, and *gB* DNA PCR targets (Ikewaki et al., 2005). We have found a high correlation between the pp65 antigenemia test and an in-house, real-time PCR targeting US17 and UL54 (Sanghavi et al., 2008). As real-time PCR is a highly sensitive test, low levels of CMV DNA can be detected during latent infection that persist for the life of the host and may not be indicative of active disease. In our study, we observed that about 1.5 to 2.5% of specimens were positive by only one PCR target and not by the other (Sanghavi et al., 2008).

CMV replicates rapidly in vivo with a doubling time of approximately 1 day (Emery, 1999). Early markers of viral replication are high viremia and rapid rate of increase in viral DNA levels (Razonable et al., 2003). Virtually all nucleic acid amplification assays are susceptible to inhibition. Endogenous (housekeeping genes) or exogenously spiked internal

control DNAs are used to normalize technical variations occurring during nucleic acid extraction and amplification processes. Blood hemoglobin has been shown to inhibit PCR amplification and quantitation of CMV DNA in a dose-dependent manner, which was corrected by normalization to an internal control (Stocher and Berg, 2002).

Detection of CMV IE and late (pp67) mRNA transcripts is indicative of active viral replication, and commercial assays such as nucleic acid sequence-based amplification have been studied for mRNA detection by some laboratories (Mengoli et al., 2004; Keightley et al., 2006). However, detection of these transcripts lacks sensitivity and is likely to miss detection of active CMV infection (Keightley et al., 2006).

Therapy

Antiviral drugs are available for both the treatment and prevention of CMV disease. Nucleoside analogs targeting CMV DNA polymerase (UL54) and protein kinase (UL97) such as acyclovir (ACV), ganciclovir (GCV), valganciclovir (VGCV), foscarnet, and cidofovir are successfully used in clinical practice. GCV, ACV, and VGCV require initial phosphorylation by viral protein kinase UL97, which is then converted into triphosphate forms by cellular enzymes. These triphosphate forms competitively inhibit viral DNA polymerases (UL54) by incorporating into the replicating DNA chains, resulting in reduction of viral DNA (Biron, 2006). Foscarnet and cidofovir do not require viral kinase for initial phosphorylation, and hence, mutations in the UL97 gene do not affect their antiviral activity. Acyclovir, which is successfully used against other herpesviruses, such as HSV and varicella-zoster virus (VZV), is less potent against CMV. Because of its toxicity, foscarnet is used as a second line of treatment for CMV disease. GCV was the first drug approved for treatment of CMV infection in immunosuppressed organ transplant recipients. It is available in intravenous and oral formulations, although because of its poor bioavailability, oral GCV is recommended for maintenance therapy. VGCV, which is a prodrug of GCV, is rapidly metabolized to the active form of GCV following intestinal absorption and has higher bioavailability of 60% when administered orally and shows promise in recent clinical trials as an effective alternative to intravenous GCV (Pescovitz et al., 2000; Czock et al., 2002; Diaz-Pedroche et al., 2005).

Prophylaxis for 3 months posttransplant can be used to prevent CMV infection in high-risk transplant recipients (Paya et al., 2004; Weng et al., 2007). VGCV is recommended for prophylaxis in CMV seropositive-donor/seronegative-recipient (D^+/R^-) solid-organ transplant recipients (Razonable and Emery, 2004; Weng et al., 2007). However, a disadvantage of this approach is the emergence of late-onset CMV disease after discontinuation of the prophylactic antiviral treatment (Limaye et al., 2004; Paya et al., 2004). Since the host is not exposed to infection during prophylaxis, it is speculated that CMV-specific immune responses are not developed and the patient is at high risk of developing severe disease when prophylaxis is stopped. Prolonged prophylaxis with a potent antiviral agent such as VGCV has a higher risk of developing late-onset CMV disease (Limaye et al., 2004). Furthermore, prolonged exposure to antiviral treatment can lead to the emergence of drug-resistant strains (Limaye et al., 2000, Limaye, 2002).

Preemptive therapy is given to prevent the progression of asymptomatic CMV infection into symptomatic CMV disease. This requires frequent monitoring of patients for CMV infection either by pp65 antigenemia or CMV DNAemia assays. Controlled asymptomatic CMV infection is said to have a favorable outcome on host immune ($CD4^+$ and $CD8^+$ T cells) reconstitution and is associated with better survival in stem cell transplant recipients on preemptive GCV therapy (Kim et al., 2006). Published guidelines for management of CMV infection and disease recommend preemptive therapy with oral GCV in liver transplant patients (Razonable and Emery, 2004). However, meta-analysis results from a recent study comparing prophylactic and preemptive therapies did not find significant differences between these two strategies in preventing CMV disease in organ transplant recipients (Small et al., 2006). Low-dose foscarnet is recommended as an alternative to GCV in preemptive treatment of CMV disease in bone marrow transplant recipients (Reusser et al., 2002; Razonable and Emery, 2004 Narimatsu et al., 2007).

There is evidence of clinical benefit of intravenous GCV treatment for symptomatic congenital CMV disease in neonates (Kimberlin et al., 2003; Michaels et al., 2003; Tanaka-Kitajima et al., 2005). While GCV therapy shows some improvement and prevention of deterioration in hearing and reduction in viral levels, it is largely limited by adverse effects such as neutropenia. A recent study presented promising results of treatment with GCV for a short period followed by oral VGCV of symptomatic CMV disease in a neonate (Meine Jansen et al., 2005). Prenatal GCV therapy in pregnant woman with CMV DNA in amniotic fluid was found to be safe and resulted in clearance of CMV DNA from amniotic fluid with no evidence of symptomatic CMV disease in the newborn baby (Puliyanda et al., 2005). Further studies are required to confirm these findings.

Fomivirsen is a small 21-nucleotide antisense RNA that specifically targets major IE transcripts of CMV, thus inhibiting viral replication (Geary et al., 2002). It is approved by the U.S. Food and Drug Administration for the treatment of CMV retinitis and is administered as an intravitreous injection.

CMV-specific hyper-immunoglobulin (CMV-Ig) in combination with GCV is commonly used for the prevention and treatment of CMV disease in organ transplant recipients (Snydman et al., 2001). A recent study showed that administration of intravenous CMV-Ig to pregnant women with evidence of CMV in amniotic fluid improved CMV-specific immune response and reduced the risk of congenital CMV disease (Nigro et al., 2005). Further studies to confirm these findings are needed.

All the above-mentioned, currently used anti-CMV drugs have similar mechanisms of action of targeting viral DNA polymerases and are limited by their side effects, such as toxicity, poor oral bioavailability, and the risk of emergence of drug-resistant strains if used for a longer duration. New anti-CMV drugs that are less toxic and have a novel mode of action are under investigation in clinical trials. A benzimidazole riboside, maribavir (1263W94), showed great potential as an anti-CMV therapeutic agent in clinical studies. It interferes with viral DNA synthesis and viral nucleocapsid egression in cell culture (Biron et al., 2002; Evers et al., 2004) by targeting UL97 protein kinase. Exhibiting negligible side effects, good bioavailability, and no in vivo emergence of resistant strains nor cross-resistance with other nucleoside analogs (GCV, cidofovir, and foscarnet) during preliminary trials, it produced a dramatic decrease in viral titer in clinical specimens from treated patients (Biron et al., 2002; Wang et al., 2003a; Drew et al., 2006). Another CMV inhibitor that is under clinical evaluation is BAY 38-4766 (Bayer Pharmaceuticals). BAY 38-4766, a

nonnucleoside inhibitor, blocks viral DNA maturation by targeting UL56 and UL89 genes (Buerger et al., 2001). The UL89 and UL56 gene products are responsible for the cleavage of viral high-molecular-weight DNA concatemers and packaging of viral DNA into procapsids. Because BAY 38-4766 targets a DNA maturation step that does not occur in normal eukaryotic cells, it is highly specific against CMV infections.

Antiviral Drug Resistance

Prolonged antiviral therapy for CMV disease carries the risk of emergence of resistant strains. Drug-resistant CMV has been reported in both bone marrow (Hamprecht et al., 2003; Marfori et al., 2007) and solid-organ transplant recipients (Limaye et al., 2000; Limaye et al., 2002) and in AIDS patients (Jabs et al., 2006; Martin et al., 2007). Mutations in the UL97 (kinase) gene or the polymerase (UL54) gene can cause resistance. Since GCV, VGCV, and ACV require UL97 kinase for initial phosphorylation, whereas cidofovir and foscarnet do not, mutations in UL97 can cause resistance only to GCV, VGCV, and ACV. However, mutations in the UL54 gene can give rise to resistance to all the anti-CMV nucleoside analogs. UL97 gene mutations are more common than the UL54 gene and are found at codons 460, 520, and 591 to 607 (Drew et al., 2001). A UL54 gene mutation is usually accompanied by mutation in the UL97 gene and can occur at multiple sites within the gene. Location of the mutation in UL54 can determine cross-resistance of GCV with cidofovir (codons 395 to 540) or foscarnet (codons 756 to 809) or both cidofovir and foscarnet (codons 978 to 988) (Chou et al., 2000; Drew et al., 2001; Baldanti et al., 2004). Persistent high CMV viremia is an indicator of drug resistance. A novel mutation in the UL97 gene was found in which deletion of codons 601 to 603 increased resistance to GCV 15-fold.

Maribavir, a UL97 kinase inhibitor, did not show evidence of cross-resistance to mutations in the UL97 and UL54 genes that cause resistance to GCV, foscarnet, and cidofovir (Drew et al., 2006). However, recent studies have shown that mutations in the UL27 gene and not UL97 gene were associated with resistance to maribavir, thus suggesting direct or indirect involvement of the UL27 gene in the mode of action of maribavir (Komazin et al., 2003; Chou et al., 2004).

Laboratory diagnosis of drug-resistant CMV strains is therefore becoming more critical for effective clinical management of CMV disease due to the use of antiviral drug prophylaxis and intensive preemptive therapy in organ transplant recipients. Phenotypic and genotypic assays are used to detect drug-resistant strains. Phenotypic assays such as plaque reduction or DNA hybridization assays measure viral growth inhibited in cell culture by increasing drug concentrations (Jabs et al., 2006). The endpoint is the drug concentration required to inhibit virus production by 50% and is reported as the 50% inhibitory concentration (Landry et al., 2000). The limitations of this assay are that it requires viable virus which is grown in prior cell culture, it is time-consuming, and some clinical isolates may exhibit variability in antiviral susceptibility between laboratories (Landry et al., 2000).

In genotypic assays, direct detection of nucleotide mutations known to cause drug resistance can be done using DNA extracted directly from patient specimens or on PCR-amplified genetic material. The mutations can be detected by either restriction fragment length polymorphism or DNA sequencing of PCR-amplified products (Eckle et al., 2003; Gilbert and Boivin, 2003; Jabs et al., 2006). Defined mutations are more commonly detected in the UL97 gene encompassing a smaller region within 400 to 650 codons. Mutations in the UL54 gene occur less frequently and encompass a larger region between 300 to 1,000 codons. Recently real-time PCR was used for rapid detection of two point mutations within the UL97 gene (Gohring et al., 2006).

Prevention

CMV is ubiquitous in distribution with high seroprevalence depending on the geographic location, making it difficult to prevent infection. Also, since it remains asymptomatic during latent infection, focus is given to preventing more serious symptomatic CMV disease in high-risk individuals. To minimize transmission, measures are done such as screening for CMV serostatus in organ donor and recipient, transfusion of blood from CMV-seronegative donors, and use of leukocyte-depleted blood for transfusion to seronegative stem cell recipients (Lipson et al., 2001). Prevention of CMV disease or infection using antiviral drugs is commonly practiced in high-risk transplant recipients using preemptive or prophylactic strategies as discussed in the earlier section on therapy.

Passive immunization to prevent CMV disease in high-risk subjects is carried out using CMV-Ig or T cells. The infusion of ex vivo expanded CMV-specific $CD8^+$ cytotoxic T-lymphocyte clones has been shown to reduce CMV viremia in high-risk bone marrow transplant recipients in patients resistant to chemotherapy (Einsele et al., 2002; Cobbold et al., 2005), although cytotoxic activity declines in patients deficient in $CD4^+$ T helper cells. A recent study showed that administration of CMV-Ig to pregnant women with primary CMV infection significantly reduced the risk of congenital CMV disease (Nigro et al., 2005).

Primary CMV infections in solid-organ transplant recipients carry the greatest risk of serious manifestations, and vaccination prior to transplant may moderate the severity of subsequent CMV disease. Live attenuated Towne and recombinant gB vaccines induced neutralizing antibodies equal to those induced after natural infection with a wild-type CMV strain (Marshall and Adler, 2003). Subunit vaccines based on gB and pp65 proteins have been evaluated to induce $CD8^+$ CTL responses. However, recently it was shown that although predominant against gB and pp65 antigens, human $CD8^+$ CTL responses target a larger number of other CMV antigens (Elkington et al., 2003; Sylwester et al., 2005), suggesting that multivalent vaccine strategies to induce broader CTL response are needed. Injection of plasmid DNA constructs from gB, pp65, and viral interleukin-10 gene products in rhesus macaques was found to be immunogenic and elicited antigen-specific humoral and cellular immune responses (Yue et al., 2007). Other promising vaccine candidates are CMV dense bodies and noninfectious enveloped particles, which are formed during CMV replication in cell cultures. These are replication-deficient forms of CMV that express a full repertoire of CMV antigens. Immunogenicity studies in mice have shown that dense bodies induce CMV-specific neutralizing antibody and CTL responses (Pepperl et al., 2000). The relatively poor immunogenicity of viral glycoproteins requires the addition of powerful adjuvants to purified gB vaccines. The immunogenicity of CMV DNA-based vaccines can be enhanced by aluminium phosphate and CpG oligonucleotides (Temperton et al., 2003). As our understanding of the CMV genome and protein functions further advances, novel vaccine candidates and strategies will evolve that give equivalent or better humoral and cellular immune responses than natural immunity.

VZV

History

VZV was first definitively recognized by isolation in cell culture in 1952 (Weller and Stoddard, 1952). The alphaherpesvirus (human herpesvirus type 3) was previously characterized by its characteristic giant cell pathology related to the common disease syndromes of childhood varicella, or chicken pox, and adult-onset zoster, or shingles. The VZV genome was sequenced in 1986 (Davison and Scott, 1986). The advent of an effective vaccine licensed in the United States for varicella in 1995 and for herpes zoster in 2006 has greatly enhanced control of this sometimes severe and fatal viral infection.

Structure, Genome, and Proteome

The VZV virion consists of an icosahedral nucleocapsid core of approximately 100 nm in diameter surrounding linear, double-stranded DNA (Cohen et al., 2007). The nucleocapsid consists of 162 capsomeres and is surrounded by a tegument that includes IE proteins involved in transactivation of viral DNA. The tegument is enveloped by a lipid bilayer membrane that is derived by budding of the nucleocapsid through various membranes of the host cell. The envelope contains virally encoded glycoprotein spikes of about 8 nm in length that project from its surface. The intact virion is about 180 to 200 nm in diameter. As an enveloped virus, the infectivity of VZV is degraded by standard physicochemical treatment, including organic solvents, heating at 56°C for 30 min, and cryopreservation for prolonged periods above –70°C.

The VZV genome of ~125,000 bp is the smallest of the eight human herpesviruses. The genome contains approximately 72 ORFs (Quinlivan and Breuer, 2006). Most of these are homologs of genes of HSV and code for similar regulatory and structural proteins (Mori and Nishiyama, 2005). Like HSV, the genome consists of UL and US segments with terminal inverted repeat regions but is predominantly found as two isomers rather than the four of HSV.

Virus Replication

Replication Cycle

Replication of VZV for the most part follows the typical course of other herpesviruses (Quinlivan and Breuer, 2006). The virion initially attaches via gB (Jacquet et al., 1998), gH (Rodriguez et al., 1993), and gI (Cohen and Nguyen, 1997) to heparan sulfate proteoglycan (Zhu et al., 1995), likely to position the virus for subsequent attachment to receptors for fusion and entry. Mannose 6-phosphate residues in the carbohydrate portions of these virion glycoproteins bind to a cation-independent mannose 6-phosphate receptor (Chen et al., 2004). Viral glycoproteins also bind to an insulin-degrading enzyme for both cell-free and cell-cell spread of virus (Li et al., 2006). After fusion of virus to the plasma membrane, tegument proteins of the virus mediate entry of the viral nucleocapsid into the cell cytoplasm, which is transported to the nucleus. There, a typical cascade of herpesvirus genes transcribing mRNA occurs, which translate into viral nonstructural, transcriptional regulator, and DNA replication proteins required for production of viral DNA and structural proteins that coat newly replicated viral DNA to form immature nucleocapsids. IE viral proteins, which are mostly found in the viral tegument, mediate the first stage of viral replication, which is transcriptional regulation of early viral protein production that is required for viral DNA synthesis. IE62 is the predominant transcriptional regulator among these and requires interactions with other viral and cellular proteins for this activity (Rahaus et al., 2003; Ruyechan et al., 2003). The third step is the production of late structural proteins of the virus. Characteristic IN inclusion bodies are formed by excess viral proteins and nucleocapsids but also include cell proteins such as heat shock protein 70 (Ohgitani et al., 1998). Like HSV, VZV DNA replication is initiated in the cell nucleus at two origin of replication loci that involve *trans*-Golgi network ORF29- and ORF51-encoded proteins (Quinlivan and Breuer, 2006). The ensuing process is similar to HSV DNA, utilizing a rolling circle and concatemeric DNA structures.

Replication of VZV is complicated by being cell type dependent. Based on infection of human fibroblasts and melanoma cells in vitro, it is known that mature nucleocapsids and enveloped particles of VZV are formed during trafficking of the immature virus particles from the nucleus through the endoplasmic reticulum to the *trans*-Golgi network (Quinlivan and Breuer, 2006). In fibroblasts, the immature virus particles acquire a temporary envelope after they are formed and bud out from the nucleus. These particles traffic to the rough endoplasmic reticulum where they lose their envelope. They then move through the Golgi to the *trans*-Golgi network (Wang et al., 2001). In this network, the nucleocapsid and tegument become sandwiched within cisterna sacs that have opposite, convex, and concave sides. The concave side of the sac is rich in viral glycoproteins, which are associated with tegument proteins and form the viral envelope, whereas the convex side contains host proteins including mannose 6-phosphate receptors that are involved in moving the particles from the *trans*-Golgi network to endosomal compartments (Wang et al., 2001). Most of the virions are degraded in these endocytic vacuoles, which relates to the well-known, limited release of cell-free VZV in vitro. The remainder of the mature viral particles transit from the endosome out of the cell (Mettenleiter, 2002). In melanoma cells, mature virions appear to form without the *trans*-Golgi network phase, move to endosomal compartments, and egress from the cell via an endocytic pathway (Harson and Grose, 1995). Moreover, gE, gB, and gH derived from the cell surface are essential for virus assembly in these cells (Maresova et al., 2005).

A key to the pathogenesis of VZV occurs after this final step of VZV replication, where the newly replicated virus spreads cell to cell and results in fusion of uninfected cells to the originally infected cell. This process is largely due to the fusogenic properties of gE, gB, gH, and gL that are present in the plasma membrane of the infected cell (Montalvo and Grose, 1987; Duus and Grose, 1996; Maresova et al., 2001). Glycoprotein E, the most predominant viral glycoprotein in the cell, acts in concert with gB as the major mediator of cell-cell fusion (Maresova et al., 2001). Likewise, gH depends on expression of gI for its maturation and function in cell-cell fusion (Duus and Grose, 1996). Cell-to-cell spread could enhance pathogenesis of the virus by preventing exposure to neutralizing antibodies.

Host Range

As an alphaherpesvirus, VZV has the general properties of variable host range, short replication cycle, rapid dissemination in cell culture, CPE, and ability to establish latency primarily in sensory ganglia (Roizman et al., 1981). Its highly restricted host range in vivo and in vitro has greatly limited studies of the pathogenesis of the virus and hindered

development of appropriate treatment and vaccines. Thus, VZV replicates efficiently in vitro only in a small variety and number of cells of human, monkey, rat, and guinea pig origin. This is further complicated by differences in VZV gene function and replication in different types of cells derived from humans, e.g., embryonic lung fibroblasts and melanoma cells. Similarly, animal models of VZV infection are very limited. Early studies showed that ganglionic cells in the guinea pig and rat can be infected with VZV, but virus cannot be reactivated (Cohrs et al., 2004). More recently, however, latent and reactivated VZV infection can be reproducibly analyzed in isolated guinea pig ganglia (Chen et al., 2003). A more complete model of VZV is infection of old-world monkeys, e.g., African green monkeys, with the simian strain of the virus, i.e., simian varicella virus (Gray, 2004). This results in varicella-like clinical symptoms including a papulovesicular rash. Moreover, the infected monkeys harbor latent virus in ganglia that can be reactivated.

VZV replicates in vitro in several types of human diploid cell lines (e.g., fetal or neonatal foreskin cells, MRC-5 fibroblasts, human embryonic lung cells, primary human kidney cells, A549 human lung carcinoma cells, melanoma cells) and B lymphoblastoid cell lines and in primary cell cultures derived from CNS tissues, including astrocytes and ganglia (Cohen et al., 2007). The virus also replicates in certain nonhuman cells, including primary rhesus monkey kidney cells and guinea pig embryo fibroblasts. The virus is highly cell associated, with little extracellular virus detected and most of the virions being replication defective. The low release of virus into the extracellular environment has been related to the interaction of newly formed virions with mannose 6-phosphate receptors, with subsequent diversion of these to lysosomes in the cell (Chen et al., 2004). This is in contrast to the extensive CPE, including giant cell formation and apoptosis (Sadzot-Delvaux et al., 1995), induced by VZV replication.

VZV Serotypes and Phenotypes

A dogma of VZV molecular biology has been the minimal genetic variability among epidemiologically distinct isolates of virus (approximately 0.1% interstrain genomic variation), with only a single serotype. However, new serotypes have been discovered that are due to naturally occurring mutations in gE (Tyler et al., 2007). These variants can spread faster in cell cultures, but it is not clear if they have significantly altered pathogenesis in vivo (Grose et al., 2004). Interestingly, there are now known to be at least four genotypes or clades of VZV that are geographically distinct (Quinlivan and Breuer, 2006). Because different genotypes are present in either warm or cool climates, it is possible that this relates to differences in the epidemiology of VZV in these regions, i.e., in temperate climates, greater than 95% of persons less than 20 years of age are infected with VZV, versus 80 to 90% infected by this age in tropical climates.

In Vivo Infection and Replication of VZV

In the past 15 years, several major advances have greatly augmented the ability to define VZV gene function. First, the availability and versatility of VZV cosmids, which are large DNA fragments of the viral genome that can be recombined to form replication-competent VZV, have allowed targeting of different ORFs with point deletion mutants (Cohen and Seidel, 1993). Second, the cotton rat provides a reproducible, small-animal model of latent VZV infection, although the virus cannot be reactivated (Sato et al., 2002; Cohrs et al., 2004). Third, the SCID/hu mouse with human skin, T lymphocyte, and dorsal root ganglia xenografts supports infection with VZV, allowing in-depth studies of VZV lytic and latency gene function and pathogenesis (Moffat et al., 1995, 1998b; Zerboni et al., 2005b; Arvin, 2006). Finally, productive infection with VZV has recently been established in ex vivo cultures of intact, human dorsal root ganglia (Gowrishankar et al., 2007). Using these in vitro and in vivo models, transcript function has been ascribed to over 40 of the 72 ORFs of VZV (Quinlivan and Breuer, 2006).

Lytic Replication

Primary VZV infection in vivo is characterized by inhalation of infected respiratory droplets or vesicular fluids, with infection of upper respiratory tract epithelial cells (Cohen et al., 2007). During the ensuing 10- to 21-day incubation period, virus spreads from these epithelial cells to T lymphocytes in localized lymphatics, particularly tonsils, possibly by transfer of virus through DCs (Abendroth et al., 2001a). These infected T cells are key to the spread of VZV to the skin. This is accomplished by homing of infected, migratory T cells across the capillary endothelium to the skin. Indeed, VZV has tropism for activated ($CD69^+$) memory ($CD45RA^-$) $CD4^+$ T cells of tonsils that express skin-homing receptors (Ku et al., 2002). Once the infected T cells reach the skin region, a battle occurs between the CPE of virus that is released by the T cells and host innate immunity, e.g., antiviral effects of IFN-α (Ku et al., 2004). Fusion of infected and uninfected epidermal cells augments cell-to-cell spread of virus, with giant cell formation and cytopathology. These epidermal cells do not have mannose 6-phosphate receptors. Thus, VZV virions do not go through an endosomal degradation phase and instead are released in high quantities from these cells.

In normal, healthy hosts, the innate immune responses limit this cell-to-cell spread and excessive cell destruction by the virus, resulting in cutaneous, intraepidermal lesions, or chicken pox. Uninfected T cells migrating through the infected dermal region can also be infected with VZV and traffic back to the circulation to spread virus to other parts of the skin, and there is also viremic spread to lymphoreticular cells in liver and spleen. Other host tropisms can include the lung alveoli, liver parenchyma, and CNS tissues. Primary VZV infection is usually limited and self-resolving, with adaptive, T-cell immunity-eliminating cells that are actively replicating virus and allowing healing of the skin vesicles. On rare occasions, however, primary infection can lead to persistent DNAemia in T cells in immunocompetent individuals with no associated residual symptoms (Vossen et al., 2005b). Primary VZV infection can also be followed by severe, disseminated disease, as well as encephalitis (Dworkin et al., 2007). It also is apparent that in the intact host, virus is not as highly restricted to infected cells as it is in vitro. This is particularly problematic in immunosuppressed individuals, where immune control of the virus is compromised and the host is unable to control primary infection or quell reactivation of latent virus (Ljungman, et al., 1986a).

Viral Latency

The hallmark property of VZV replication in vivo is establishment of a permanent latent state in several types of sensory neuronal cells, including trigeminal, dorsal root, and cranial ganglia (Levin et al., 2003; Zerboni et al., 2005b). Although nonneuronal cells proximate to neuronal cells

have been reported to harbor latent VZV (Croen and Straus, 1991), viral DNA was found only in neuronal cells of 34 of over 2,000 human ganglia and none in satellite, nonneuronal cells (Levin et al., 2003). Guinea pigs and rats, in particular, the cotton rat (Sato et al., 2002), can be latently infected with VZV, mimicking a limited degree of latency in humans. Most of the VZV transcripts expressed during latency in human neurons also are expressed in the latently infected cotton rat. Notably, however, VZV cannot be reactivated from latency in rat cells either in vivo or in vitro.

The latent state of VZV is unique among herpesviruses in that it expresses different RNA transcripts and is present in unusual anatomic and cellular sites compared to other herpesviruses, particularly HSV (Kinchington, 1999). Although most of the VZV ORFs studied to date have no role in latency (Quinlivan and Breuer, 2006), establishment of latency is controlled in part by ORF63, an IE transcriptional regulator of VZV, as shown in human neural cells in vitro (Baiker et al., 2004) and in the cotton rat model (Cohen et al., 2005). Indeed, ORF63 is the most frequently detected and abundantly transcribed VZV gene in human ganglia (Cohrs and Gilden, 2007). ORF63 encodes a tegument protein that has a significant function in both lytic and latent VZV infection by regulating the transcription of several other VZV proteins involved in VZV replication. Other ORFs that are transcribed during latency are ORF4, ORF21, ORF29, ORF62, and ORF66 (Cohrs et al., 2004; Cohen et al., 2005). An average of 4% of neuronal cells in the trigeminal ganglia harbor latent VZV (Wang et al., 2005a; Wang et al., 2005b). The key to VZV establishing and maintaining VZV latency in neuronal cells is localization of the transcribed regulatory proteins in the cytoplasm of latently infected neurons rather than their usual location in the nucleus during lytic replication of VZV (Lungu et al., 1998). In addition, it has been suggested that host CTL reactivity to latency proteins such as ORF63 inhibits reactivation of latent virus (Sadzot-Delvaux et al., 1997). The mechanisms of reactivation of latent VZV have not been elucidated but presumably involve modulation of these regulatory proteins.

Reactivation of latent VZV probably occurs throughout the life of the host after primary infection but is controlled by immune mechanisms and normally remains subclinical. Indeed, VZV can be detected by PCR amplification of viral DNA in the blood of older, asymptomatic individuals (Devlin et al., 1992; Schunemann et al., 1998) as well as immunocompromised, asymptomatic patients (Wilson et al., 1992; Mainka et al., 1998). Reactivation of latent VZV in dorsal root ganglia occurs primarily in elderly persons, presumably due to a progressively lower T-cell immune response to the virus during older age. This results in either a subclinical outcome, or clinical syndrome, including a chronically painful zoster without rash (zoster sine herpete) or overt zoster with a classic, thoracic dermatoform vesicular rash (shingles). These clinical conditions can be much more severe and life-threatening in immunocompromised hosts, such as organ and tissue transplant recipients, cancer patients, and persons infected with HIV-1.

Immune Response to VZV

Innate and Adaptive Immunity

Primary VZV infection induces both innate and adaptive immune reactivity. In vitro studies suggest that VZV activates inflammatory cytokine production via TLR2 as part of the innate immune response (Wang et al., 2005a). NK cells also are likely to be involved in control of VZV infection, as children with severe varicella lack circulating NK cells (Vossen et al., 2005a).

Primary VZV infection elicits a humoral immune response to the virus characterized by circulating IgG, IgM, and IgA that include neutralizing antibodies directed against viral glycoproteins and regulatory proteins (Arvin, 1996). The levels of IgM antibodies subside within a few months, whereas IgG antibodies persist for the life of the host and may be of importance in prevention of reinfection and reactivation of latent virus. Anti-VZV IgG is likely to be important in control of latent VZV, given the well-established association of circulating IgG levels in infected hosts, which decline in titers associated with age and increased risk for reactivated infection (Arvin, 1996). There also is loss of VZV-specific antibodies over time in children infected with HIV-1, which is not reversed even after suppression of HIV-1 infection during antiretroviral therapy (Bekker et al., 2006a).

Although VZV-specific IgM and IgG responses are not associated with control of primary viremia or latent virus (Arvin, 1996), the presence of VZV-specific IgG is associated with milder disease in stem cell transplant recipients (Onozawa et al., 2006). Moreover, the level of serum antibody to VZV as detected by enzyme-linked immunosorbent assay (ELISA) correlates with the protective efficacy of the vaccine (Li et al., 2002). Indeed, the best evidence that antibodies to VZV participate in control of VZV infection is that administration of VZV immune globulin (VZIG) has been successful as prophylaxis for preventing VZV disease in immunosuppressed individuals (Weinstock et al., 2004) and in other at-risk persons exposed to VZV, e.g., pregnant women (Arvin, 2006).

T-cell immunity is considered of paramount significance in control of both primary and reactivated VZV infections. Lower levels of anti-VZV T-cell responses are associated with increased risk for severe primary and reactivated infections (Arvin, 1996). Both $CD8^+$, MHC class I-restricted CTL reactivity, and MHC class II-restricted $CD4^+$ helper responses specific for VZV structural and regulatory proteins are associated with resolution of primary VZV infection and persist for years in the memory cell population (Asanuma et al., 2000). Notably, $CD8^+$ CTL immunity targets IE proteins, IE62 (Arvin et al., 2002; Frey et al., 2003) and IE63 (Sadzot-Delvaux et al., 1997), IE proteins encoded by ORF4 (Arvin et al., 2002) and ORF10 (Arvin et al., 2002), and the early protein coded by ORF29 (Arvin et al., 2002). Differential CTL activity to these proteins could impede VZV pathogenesis by lysing cells during the early, pre-virion production phase of VZV replication. Likewise, $CD4^+$ T-cell responses are targeted to ORF4 (Jones et al., 2006) early after primary infection as well as to the late glycoprotein gI (Malavige et al., 2007). T-cell responses to VZV are a highly sensitive measure of reexposure to the virus in adults, as increases in $CD4^+$ T cells specific for VZV have been found in VZV-immune adults exposed to persons with varicella, in the absence of symptoms and detectable viral DNAemia (Vossen et al., 2004). Interestingly, $CD4^+$ memory T-cell responses to VZV antigens may not persist in females as well as they do in males (Klein et al., 2006), which needs to be addressed in more extensive studies.

Immune Evasion

Replication of VZV results in cellular alterations that may affect the ability of the host to control the viral infection (Abendroth and Arvin, 2001). Notably, the virus encodes a

glycoprotein, gE, on the cell surface which, when complexed with gI, resembles the Fc γRII receptor (Litwin and Grose, 1992), which could allow VZV to evade host antibody. VZV also down-regulates MHC class I by inhibiting transport of MHC class I molecules from the Golgi apparatus to the cell surface, which has been related to ORF66 function (Abendroth et al., 2001b), which could impede VZV-specific $CD8^+$ CTL activity. The virus also impedes IFN-γ enhancement of MHC class II expression in VZV infection by inhibiting transcription of interferon regulatory factor 1 and the MHC class II transactivator (Abendroth et al., 2000), which could decrease $CD4^+$-T-cell reactivity. In fact, VZV has the unique ability to sequester NF-κB in the cytoplasm of infected cells, possibly using this as a pathway that could hinder host antiviral responses (Jones and Arvin, 2006). These cumulative results suggest that VZV infection can inhibit innate immunity and adaptive $CD8^+$- and $CD4^+$-T-cell-mediated responses.

Epidemiology and Disease Manifestations

Varicella

VZV infection is present throughout the world, with greater prevalence of varicella during inclement months due to spread of virus indoors by respiratory secretions to close contacts (Wharton, 1996; Arvin, 1996). Prior to the advent of a vaccine, over 90% of adults in the United States were immune to VZV. The prevalence is lower in less-developed countries, particularly in tropical regions. The virus infection is spread by respiratory secretions and is therefore almost ubiquitous in children, resulting in a self-limiting, predominantly symptomatic illness that rarely recurs. Prodromal symptoms of fever and malaise occur during the prolonged, 2-week incubation period prior to outbreak of the classic, maculopapular rash. The rash occurs primarily on the scalp and trunk. These vesicular lesions crust over within 1 to 2 days but do not fully heal for up to 3 weeks. Complications are uncommon in immunocompetent hosts but can include opportunistic bacterial infections of the skin lesions and lungs, thrombocytopenia, varicella pneumonia, and encephalitis. Additionally, congenital infection can be disseminated and quite severe, as can postnatal infections (Kurlan et al., 2004), especially if the infant has a VZV-seronegative mother and therefore does not have maternal antibodies (Gardella and Brown, 2007).

Immunocompromised patients, particularly children with untreated HIV-1 infection or underlying cancers, may have a more severe varicella syndrome (Gershon et al., 1997; Kavaliotis et al., 1998). This is usually characterized by greater numbers of lesions and systemic infection of visceral tissues and high mortality. This is a major problem in children with immunosuppression related to leukemia or bone marrow transplantation (Leung et al., 2000; Maltezou et al., 2000; Hackanson et al., 2005). Notably, visceral VZV complications also can be atypical in these patients, in some cases with no or few skin lesions (Mantadakis et al., 2005; Leveque et al., 2006). Furthermore, treatment with immunosuppressive steroids, which are used to ameliorate the pain of zoster in nonimmunosuppressed individuals (Dworkin et al., 2007) can enhance the pathogenesis of VZV in leukemia patients (Hill et al., 2005). Of importance is that varicella vaccine is safe, immunogenic, and cost-effective in preventing severe disease in pediatric organ transplant recipients (Olson et al., 2001; Chaves Tdo et al., 2005; Weinberg et al., 2006) and HIV-1-infected children (Levin et al., 2006; Bekker et al., 2006b).

Zoster

Herpes zoster occurs in about 20% of nonimmunocompromised persons who have had primary VZV infection (Dworkin et al., 2007). It is a manifestation of reactivated latent VZV infection occurring predominantly in immunocompetent persons over 45 years of age. The pathology usually follows an anatomic route around the torso along the dorsal ganglia, as virus spreads cell to cell along the neurons to epithelial cells in the skin. This results in vesicular lesions similar to those of varicella. The lesions are controlled by host immunity, particularly anti-VZV CTL, and ultimately resolve within several weeks. A significant common complication of zoster is postherpetic neuralgia (Gilden et al., 1994; Schmader, 1998; Niv and Maltsman-Tseikhin, 2005). This is a chronic, debilitating pain that begins approximately 1 month after onset of skin lesions and can persist long after the resolution of the overt signs and symptoms of zoster. The pain may be due to destruction of neurons by VZV infection. The incidence of this syndrome increases with older age. Other rare but severe complications of zoster in immunocompetent individuals include meningitis (Mogensen and Larsen, 2006), encephalitis (Gnann, 2002; Koskiniemi et al., 2002), bullous erythema multiforme or Stevens-Johnson syndrome (Weisman et al., 1998), hemolytic anemia (Terada et al., 1998), Ramsey-Hunt syndrome (Murakami et al., 1998), and ophthalmic disease (herpes zoster opthalmicus), including optic neuritis, perivasculitis, and atypical necrotizing retinopathy (Garweg and Bohnke, 1997; Wenkel et al., 1998; Lau et al., 2007).

Immunocompromised individuals such as adults with HIV-1 infection and certain cancers are at risk for development of serious and sometimes life-threatening zoster (Weller, 1983; Glesby et al., 1995; Gnann, 2002). The incidence of zoster in HIV-1-infected adults not on potent antiretroviral therapy is approximately 15 times greater than in HIV-1-seronegative persons (Buchbinder et al., 1992). As with varicella, the number of lesions is usually higher and the lesions last longer in these immunocompromised patients. Complications include pneumonia, encephalitis, and necrotizing retinopathy (Cohen and Grossman, 1989; Miller et al., 1997). The incidence and severity of opportunistic infections in HIV-1-infected persons significantly declined after 1997 with the advent of potent antiretroviral drug therapy (Jacobson and French, 1998). Indeed, the incidence rates for herpes zoster in children has decreased from 2.1 to 1.1 after widespread use of potent antiretroviral therapy in the United States (Gona et al., 2006). However, several small studies have found a perplexing increase in the incidence of herpes zoster shortly after initiation of antiretroviral therapy (Aldeen et al., 1998; Domingo et al., 2001; Patel et al., 2006; Venkataramana et al., 2006). This is associated with an increase in the number of circulating $CD8^+$ T cells, suggesting that these are involved in the disease process. This seemingly enigmatic, early outcome of antiretroviral therapy could be linked to the emerging immune reconstitution inflammatory syndrome. The syndrome is defined as a decline in clinical or laboratory parameters concurrent with an increase in $CD4^+$-T-cell counts and decrease in HIV-1 viral load. It usually can be controlled by changing the antiretroviral regimen or use of corticosteroids.

Diagnosis

The importance of laboratory determination of VZV infection is highly dependent on the nature of the clinical illness. Thus, laboratory diagnosis of VZV infection is not

recommended for conventional, uncomplicated varicella or zoster syndromes in otherwise healthy children and adults. This is based on recognition of the classic signs and symptoms of varicella, the backdrop of endemic varicella in the geographic location or known exposure to someone with varicella, and the lack of indicators of underlying immune deficiency. This is also prudent for the management of health care costs.

Nevertheless, there are bona fide circumstances where a laboratory-based diagnosis of varicella is appropriate. These are, first, the presence of immunosuppressive conditions in the patient, such as cancer or HIV-1 infection; second, the occurrence of varicella in a neonate, a presumed immune person, or a person who has been vaccinated; third, zoster in a young person; fourth, occurrence of significant clinical complications of VZV infection or of unusual syndromes that have been related to VZV infection; fifth, when atypical lesions occur that, for example, could be due to HSV infection; sixth, to distinguish VZV lesions from smallpox lesions in the event of a bioterrorism threat. In these cases, a laboratory diagnosis of VZV infection can be essential to determining the clinical prognosis and making therapeutic decisions, i.e., when to start, stop, or change antiviral therapy.

Direct Identification of CPE

A traditional method for determining VZV infection is to identify IN inclusions and giant cells in scrapings from the base of a fresh vesicular lesion that have been stained with either Tzanck, Giemsa, or hematoxylin and eosin preparations (Woods and Walker, 1996). Such histological diagnoses are complicated, however, by the identical inclusion bodies formed in HSV-infected cells. This becomes of major significance in determining disease prognosis and proper antiviral drug treatment regimens. Staining of cell smears with colloidal gold complexed to anti-VZV IgG also has been used to identify VZV directly in cell smears by electron microscopy (Vreeswijk et al., 1988).

Culture of Infectious Virus

A conventional method for diagnosing VZV infection has been identification of characteristic CPE consisting of small clusters of ovoid cells in monolayers of cultures of human fibroblasts (Gershon et al., 2007). The cell lines of choice for isolation of VZV are human diploid cell lines HF, MRC-5, and A549. This procedure, however, is fraught with problems, including the need of fresh vesicle fluid, the relatively long time to positivity (usually 7 to 10 days), and the loss of the cell cultures to microbial contamination. An improvement in the culture technique that is now the standard method in most diagnostic laboratories is inoculation of specimens into fibroblast cultures (e.g., MRC-5) that have been grown on glass coverslips in shell vials. The coverslips are fixed with acetone after 3 and 6 days of culture and stained with fluorescein isothiocyanate-conjugated monoclonal IgG antibody specific for VZV glycoprotein (Gleaves et al., 1988; West et al., 1988). Positive cells have cytoplasmic, apple-green fluorescence. This procedure is more sensitive and usually more rapid for identification of VZV than standard tube cultures.

Direct Identification of Viral Antigens

A method for more timely diagnosis of VZV infection is direct staining of vesicular epithelial cell smears with fluorescent dye-conjugated, monoclonal antibodies to VZV IE and glycoprotein antigens (Gleaves et al., 1988; Coffin and Hodinka, 1995; Dahl et al., 1997; Chan et al., 2001). This procedure can be done within hours of obtaining the specimen. The method is highly dependent on the quality of the specimen, as too few epithelial cells are a common problem. The procedure is more sensitive than conventional tube cell culture (97.5% versus 49.4%) (Coffin and Hodinka, 1995) and is less costly, particularly for labor.

Molecular Diagnosis

Molecular detection of VZV DNA by in situ hybridization and PCR is sensitive and specific for diagnosis of VZV infection in vesicle fluids and skin biopsy specimens (Annunziato et al., 1996; Beards et al., 1998). These molecular procedures are gradually replacing antigen detection methods for diagnosis of VZV infections as they are becoming more cost-effective for the routine laboratory. Rapid, differential diagnosis of zoster and HSV lesions, which is important for prognosis and treatment, can be done using PCR (Rubben et al., 1997). Detection of VZV infection in CSF directly by PCR and hybridization, and indirectly by measure of anti-VZV antibodies, is of value in the diagnosis and treatment of varicella and zoster encephalitis (Puchhammer-Stockl et al., 1991; Burke et al., 1997; Markoulatos et al., 2001; Weidmann et al., 2003). A problem is that PCR also detects VZV DNA in the CSF of persons with subclinical virus reactivation (Cinque et al., 1997).

Detection of VZV DNA in oropharyngeal swabs of patients with zoster sine herpete (Furuta et al., 1997) and in aqueous and vitreous humor of patients with retinitis (Danise et al., 1997; Knox et al., 1998; Zhang et al., 2003) is used for diagnosis of these syndromes. Additionally, PCR has been used to detect VZV in amniotic fluids of mothers with incident varicella in early pregnancy (Mouly et al., 1997). This may be of benefit in diagnosing and possibly treating congenital varicella.

The technical ease and specificity of detection of VZV by PCR has been improved by the advent of real-time PCR. Using primers for different targets within VZV ORFs, detection of VZV DNA by real-time PCR has been shown to be more sensitive and specific and technically superior to culture or immunofluorescence for dermal and CNS specimens (Espy et al., 2000). Addition of phocine (seal) herpesvirus to the clinical samples as an internal control prior to extraction allows monitoring for accurate control of the whole process (van Doornum et al., 2003; Stranska et al., 2004). An extension of this technology has been the detection of multiple human herpesviruses in the same clinical specimen, termed "multiplex PCR." Thus, DNA from VZV, HSV-1 and HSV-2 (Hobson-Peters et al., 2007), and in some cases, also CMV, Epstein-Barr virus (EBV), and human herpesvirus 6 (Markoulatos et al., 2001; Stocher et al., 2004), as well as an internal control, can be simultaneously detected by real-time PCR in a single sample extracted from a clinical specimen. This may be of value in the care of patients who are susceptible to multiple herpesvirus infections due to immunosuppressive conditions. Finally, an automated, DNA microarray PCR method has been developed for high-throughput detection of multiple herpesviruses, including VZV, in clinical samples (Foldes-Papp et al., 2004).

Real-time PCR has been used to quantitate levels of VZV DNA in blood (Ishizaki et al., 2003; Kalpoe et al., 2006). This should be useful in monitoring patients at high risk for severe VZV infection, such as stem cell transplant recipients. In some cases, VZV DNA can be detected in blood several days before the onset of clinical symptoms of VZV infection (Ishizaki et al., 2003), thus allowing for early initiation of therapy to preempt the onset of disease.

Serum Antibody

Serologic assays for detection of anti-VZV IgG and IgM antibodies have been in use for many years (Arvin, 1996). They are of limited value in diagnosis due to the need for matched acute- and convalescent-phase or chronic samples, long turn-around time, indirect measure of infection, and false positives for IgM. The determination of VZV antibody is important, however, when there is a question regarding the immune status of a person, particularly in immunosuppressed patients, and for determining the immunogenicity of VZV vaccination (Sauerbrei and Wutzler, 2006). They are also of critical value in epidemiologic studies of VZV, where they have been adapted to use with oral fluid specimens (Talukder et al., 2005).

The accepted standard in the field for assessing immunity to VZV is the fluorescent-antibody-to-membrane-antigen (FAMA) test (Krah, 1996). This assay detects antibodies specific for viral envelope glycoproteins using human fibroblasts infected for several days with a defined infectious dose of VZV Oka strain that produces sufficient surface glycoproteins. The infected cells are fixed with glutaraldehyde in microtiter wells and mixed with serial dilutions of the patient's serum (Sauerbrei et al., 2004). These cells are then stained with fluorescein isothiocyanate-labeled rabbit anti-human IgG and Evans blue dye. The percentage of infected cells at each dilution of serum is determined by fluorescence microscopy. Titers of ≥2 are considered positive. VZV antibody standards are available to validate the assay. Unfortunately, this assay is best done using in-house procedures, as commercial FAMA kits have been unreliable. Improved, indirect immunofluorescence assays have been reported to be more sensitive and less labor-intensive to perform than FAMA (Sauerbrei et al., 2004; Sauerbrei and Wutzler, 2006). Commercial ELISAs are also available for detecting and measuring VZV antibodies (Maple et al., 2006).

Therapy

Drug prophylaxis and treatment for VZV infection have improved dramatically during the last 30 years. There are now several licensed antiviral drugs in the United States effective against VZV infection (Dworkin et al., 2007). These replaced the first generation anti-VZV drugs IFN-α (Merigan et al., 1978) and vidarabine (Shepp et al., 1986) for treatment of VZV.

ACV

2-Amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6H-purin-6-one (Zovirax; GlaxoSmithKline) is a purine nucleoside analog that inhibits the viral thymidine kinase (TK) (Villarreal, 2003). It is recommended for VZV infections in persons with underlying immunodeficiencies, such as HIV-1 infection, neoplasia, and organ or tissue transplantation, neonates, and persons with severe clinical complications. The drug has greatly improved the prognosis of VZV infection in such immunocompromised children and adults, but does not eliminate latent virus infection. ACV is converted to ACV monophosphate by the viral TK. This nucleotide analog is then converted into a diphosphate and finally into the triphosphate form of ACV. The triphosphate ACV halts VZV replication by competitive inhibition and inactivation of the viral DNA polymerase and by termination of DNA chain elongation. ACV is used for both acute varicella and zoster, particularly in immunocompromised hosts. There are several variations in dosage recommended based on the disease syndrome, especially in immunocompromised patients with chronic, recurrent disease (Dworkin et al., 2007). The drug may be most effective in quelling serious diseases such as retinitis in immunocompromised patients when given in combination with other anti-VZV drugs (Moorthy et al., 1997). ACV does not inhibit postherpetic neuralgia (Acosta and Balfour, 2001).

ACV has been used successfully as prophylaxis for varicella in at-risk, exposed persons (Lin et al., 1997; Ogilvie, 1998; Yoshikawa et al., 1998; Thomson et al., 2005). Administration of low-dose ACV for the first 6 months after allogeneic bone marrow transplantation is well tolerated and reduces the incidence of VZV disease (Ljungman et al., 1986b; Selby et al., 1989). This antiviral prophylactic effect can be prolonged by increasing the period to 12 months after transplantation (Boeckh, 2006; Boeckh et al., 2006; Weinstock et al., 2006).

Resistance of VZV to acyclovir can occur during chronic administration by mutations in the viral TK gene (Jacobson et al., 1990; Reusser, 1996; Fillet et al., 1998) and requires a switch to another antiviral regimen.

Famciclovir

2-[2-(2-Amino-9H-purin-9-yl)ethyl]-1,3-propanediol diacetate (Famvir; Novartis) is the oral, prodrug form of penciclovir that has greater bioavailability against VZV infection after oral administration (Cirelli et al., 1996; Simpson and Lyseng-Williamson, 2006). Famciclovir is an acyclic guanine derivative that is rapidly transformed into the active antiviral form of penciclovir. The drug is phosphorylated by viral TK to penciclovir monophosphate and then by cellular kinases to the triphosphate form of penciclovir. It inhibits the VZV DNA polymerase by competing with deoxyguanosine triphosphate. Penciclovir has similar efficacy to ACV, but has more prolonged antiviral activity in the host. Based on its high antiviral potency and favorable tolerance, famciclovir is a first-line option for treatment of herpes zoster. The drug enhances healing of acute zoster and decreases the pain of postherpetic neuralgia.

Valacyclovir

L-Valine, 2-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methoxy]ethyl ester, monohydrochloride (valacyclovir [Valtrex; GlaxoSmithKline]) is the hydrochloride salt of the L-valyl ester of ACV (Villarreal, 2003; Enright and Prober, 2003). This prodrug is rapidly converted to ACV and has greater bioavailability. It also decreases the pain of postherpetic neuralgia.

Foscarnet

Phosphonoformic acid, trisodium salt (foscarnet [Foscavir; AstraZeneca]) is an organic analog of inorganic pyrophosphate. It inhibits herpesvirus replication at the pyrophosphate binding site of the viral DNA polymerase (Villarreal, 2003). It is effective for treatment of ACV-resistant VZV. It has been used as a second-line treatment for ACV-resistant VZV (Balfour et al., 1994; Boivin et al., 1994). Foscarnet-resistant VZV has been reported, with the likely mutations responsible being in the viral DNA polymerase (Visse et al., 1998).

Brivudine

Brivudine, (e)-5-(2-bromovinyl)-2′-deoxyuridine (Helpin, Berlin-Chemie), is a halogenated thymidine nucleoside analog that is converted to its diphosphate and triphosphate derivatives, which inhibit viral DNA polymerase and are incorporated into viral DNA (Andrei et al., 1995). It is highly specific for VZV DNA and more potent against VZV

in vitro than ACV or penciclovir. In clinical trials, it has been shown to be well tolerated, to shorten the replication phase of VZV, and to inhibit formation of new vesicles better than ACV (Wassilew and Wutzler, 2003). As of this writing, brivudine is approved for use in Austria, Belgium, Germany, Greece, Italy, Luxembourg, and Spain.

BCNAs

Bicyclic pyrimidine nucleoside analogues (BCNAs) are relatively new inhibitors of VZV infection that have chemistry similar to that of brivudine (Balzarini and McGuigan, 2002). The promising new drug family has many properties that are equal or superior to other anti-VZV agents, including high potency and selectivity for VZV with no other known antiviral specificity, as well as good oral bioavailability and low toxicity. Various derivatives of BCNAs are currently undergoing preclinical development (Andrei et al., 2005; McGuigan and Balzarini, 2006).

The requirement by VZV of both viral and cellular kinases for replication has been exploited as a target for a new class of purine derivatives that inhibit cyclin-dependent kinases, roscovitine and purvalanol (Moffat et al., 2004). These agents are novel as antiviral drugs in that they act on cellular cyclin-dependent kinases that are required for VZV replication, in both rapidly dividing and nondividing cells (Taylor et al., 2004). Other attractive properties of these agents are their low toxicity and antiviral kinase activity.

VZIG

VZIG (American Red Cross) is a high-titered, anti-VZV antibody preparation that is widely used as postexposure prophylaxis to prevent VZV infection in certain patients at high risk of serious disease (Slifkin et al., 2004). Unfortunately, the production of VZIG was discontinued in 2004. To replace VZIG, VariZIG (Cangene) is now available under an investigational new drug application and expanded access protocol. It is recommended by the CDC for use in immunocompromised patients, neonates whose mothers have signs and symptoms of varicella around the time of delivery, premature infants who are at risk for VZV infection due to exposure during the neonatal period and lack of anti-VZV immunity in their mothers, and pregnant women (Centers for Disease Control and Prevention, 2006).

Prevention

Varicella Vaccine

In 1995, the first human herpesvirus vaccine, Varivax (Oka; Merck) was licensed for use in the United States (Arvin and Gershon, 1996; Grose, 2005). In 2005, a vaccine consisting of live attenuated measles, mumps, rubella, and varicella viruses (MMRV [ProQuad; Merck]) was licensed in the United States (Centers for Disease Control and Prevention, 2005). The vaccine is safe in immunocompetent persons, with only rare complications, and is highly immunogenic, with over 90% of vaccinated children developing antibodies to the virus (Hambleton and Gershon, 2005). Antiviral immunity induced by the vaccine persists to adulthood (Ampofo et al., 2002). The universal childhood vaccination policy in the United States since 1995 has resulted in a significant decrease in the incidence of varicella and associated severe complications (Grose, 2005). The virus used for the vaccine was originally isolated by Takahashi and colleagues in the mid-1970s from a child with varicella and then passaged in human and guinea pig cells to achieve a live attenuated preparation (Takahashi et al., 1974). The vaccine strain has many point mutations that result in a reduced capacity to replicate in skin epithelial cells (Moffat et al., 1998a). Studies using Oka vaccine recombined with various regions of the parental strain of Oka indicate that this attenuation of the vaccine strain is related to several different ORFs (Zerboni et al., 2005a), particularly ORF62 (Cohrs et al., 2006).

Trials in the United States indicated that the Merck vaccine is safe, induces a long-lasting T-cell immunity to VZV (Gershon and Steinberg, 1989; Zerboni et al., 1998), is highly effective in preventing primary varicella in normal children (Weibel et al., 1984), and decreases the incidence of zoster in leukemic children (Hardy et al., 1991; Lawrence et al., 1988). A new formulation of the varicella vaccine has been developed as a combined measles, mumps, rubella, and varicella vaccine (MMRV [Proquad; Merck]) and is safe and immunogenic in children (Kuter et al., 2006; Lieberman et al., 2006). The vaccine is currently being proposed for use for prevention of varicella in the United States in children less than 13 years of age in a 2-dose format, with the first dose given at 12 to 15 months of age and the second dose at 4 to 6 years of age. The vaccine is also recommended, with certain qualifications and contraindications, for HIV-1-infected children greater than 12 months of age; for postpartum, nonimmune women; during outbreaks of varicella; for susceptible healthcare workers; and for family contacts of immunocompromised people.

The Oka/Merck vaccine also has been used as a therapeutic regimen to prevent or at least alleviate the severity of complications of VZV infection (Levin, 2001). This is based on the correlation of decreased VZV-specific T-cell immunity with increased risk of zoster (Arvin et al., 1996). As proof of efficacy of this approach, cellular and humoral immune responses to VZV have been boosted by vaccination of elderly patients with the Oka vaccine (Berger et al., 1998; Levin et al., 1998). The risk for zoster appears to be decreased in leukemic children by either exposure to natural varicella or by vaccination with Oka (Gershon et al., 1996). Additionally, administration of a heat-killed VZV vaccine after hematopoietic stem cell transplantation reduced the severity of zoster in leukemic children (Redman et al., 1997) and the risk for zoster in persons with non-Hodgkin's or Hodgkin's lymphoma (Hata et al., 2002).

There are concerns about the live attenuated Oka/Merck vaccine, however. The vaccine can cause disease, particularly in immunosuppressed persons. The vaccine strain has been transmitted to healthy household contacts, resulting in overt skin lesions (LaRussa et al., 1997). A persistent problem has been breakthrough infection with wild-type virus (Takayama et al., 1997; LaRussa et al., 2000). Some of this breakthrough is likely due to the lack of 100% immune response in the population to the single-dose vaccine. Other risk factors are a several-year interval since the vaccination and receiving the vaccination within the first year of life (Grose, 2005). Of importance is that evidence so far suggests that, even though the vaccine establishes latency, it does not commonly reactivate and cause zoster (Arvin et al., 1996).

A major concern with the vaccine relates to the observation that immunity to VZV can be boosted in adults harboring latent infection by exposure to persons with varicella, thereby decreasing their risk for herpes zoster. Thus, a decrease in the incidence of varicella due to the vaccine could limit this protective effect. There is no evidence, however, for an increase in the incidence of vaccine-associated herpes zoster since the use of the vaccine (Jumaan et al., 2005).

Furthermore, use of a second, booster dose of the vaccine could enhance its immunogenicity and prevent the potential loss of immunity to VZV years after vaccination (Shinefield et al., 2005).

Zoster Vaccine

In 2006, a live attenuated vaccine for prevention of herpes zoster (Zostavaz; Merck) was licensed in the United States. This was based largely on the favorable outcome of a large clinical trial showing a decrease in the incidence of herpes zoster and postherpetic neuralgia in adults 60 years of age or older (Oxman et al., 2005). The vaccine prevented shingles in 51% and postherpetic neuralgia in 67% of the study participants. The vaccine was most effective in persons 60 to 69 years of age, but it also decreased the severity of incident zoster in people 70 years or older. The zoster vaccine is essentially a higher-titered version of the varicella Oka vaccine, which is estimated to have about 14 times greater potency at the minimum dose. Presumably, this potency is required to elicit a sufficient cellular immune response to inhibit the reactivation of latent VZV or its pathogenic effects.

EBV

Historical Perspective

EBV was discovered four decades ago during a search for the etiology of Burkitt's lymphoma (BL) affecting children in sub-Saharan Africa. The high incidence of lymphomas was initially described by Dennis Burkitt, who suspected the involvement of an infectious, possibly arthropod-borne, agent (Burkitt, 1958, 1963). Cells obtained by Burkitt from the tumors of Ugandan children were sent via air freight to the laboratory of Epstein and Barr at Middlesex Hospital in London. After surviving for several days at ambient temperatures, some of the specimens contained viable lymphoblasts which grew in culture and harbored virus particles detected by electron microscopy (Epstein et al., 1964). The BL explants grew into cell lines which contained antigens detectable with sera from BL patients (Henle and Henle, 1966). This provided the basis for a seroepidemiologic study leading to the discovery of EBV as the causative agent of IM (Henle et al., 1968a; Niederman et al., 1970). Ironically, the first clue to the connection between EBV and IM came from serum obtained from a research assistant in the laboratory of Walter and Gertrude Henle who had been conducting the seroepidemiologic surveys. Sera obtained from the assistant before a classic episode of IM were negative, while sera obtained immediately after IM were positive (Henle et al., 1968a).

Initial efforts to grow the virus by cocultivation of irradiated BL cells with human neonatal lymphocytes led to the next surprising discovery (Henle et al., 1967; Miller et al., 1969). Instead of causing cytopathology, the virus induced neonatal B lymphocytes to proliferate indefinitely in vitro. The efficiency of the process was so high that a limiting dilution endpoint assay for immortalization of B cells became the standard assay for measuring the titer of biologically active virus in a culture supernatant. Research over the last 4 decades has focused on the immortalizing phenotype of EBV principally because this activity has been proposed as the mechanism underlying the pathogenesis of the lymphoprolferative diseases with which the virus has come to be associated.

Biology and Pathogenesis

Epstein-Barr virus (human herpesvirus 4) is the prototype for the *Lymphocryptovirus* subfamily of the gammaherpesviruses (Roizman, 1996). EBV can attach to and infect either B lymphocytes or epithelial cells. At least eight virus envelope proteins have been implicated in EBV entry, and their roles vary depending on the cell type being infected (Hutt-Fletcher, 2007). For entry into B cells, the gp350/220 mediates binding to cell surface CD21 (Szakonyi et al., 2006). Incubation of virus with antibodies to gp350/220 or soluble CD21 blocks virus binding and neutralizes B-cell infection. Recombinant EBV lacking gp350/220 still immortalizes B cells but with much-reduced efficiency (Janz et al., 2000). This has prompted further investigation of roles of other envelope glycoproteins (gHgL, gB, and gp42) that turn out to be both necessary and sufficient for envelope-cell membrane fusion within endocytic vesicles (Hutt-Fletcher, 2007). Attachment to epithelial cells appears to be largely dependent upon the gHgL complex, and recombinant virus lacking gHgL cannot infect epithelial cells or B cells (Molesworth et al., 2000; Oda et al., 2000). In B-cell infections, gp350/220 mediates cross-linking and capping of CD21 triggers an initial activating signal that leads to blast transformation, homotypic cell-cell adhesion, expression of B-cell activation antigens, and production of cytokines (Gordon et al., 1986; Tanner et al., 1987, 1996). Within 3 to 4 h of attachment, the linear 172-kb DNA genome is delivered to the nucleus and coated with host cell histones. The virus then initiates the immortalization program, expressing a subset of its genes that drive proliferation while preventing apoptotic death. Over the next 48 to 96 h six Epstein-Barr nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP), two membrane proteins (LMP1 and LMP2), and two short RNAs (EBER1 and EBER2) are made (Kieff, 1996). Of these genes, six (EBNA1, EBNA2, EBNA3A, EBNA3C, EBNA-LP, and LMP1) have been directly implicated in immortalization by knockout recombinational analyses and/or inhibition with antisense oligonucleotides (Hammerschmidt and Sugden, 1989; Cohen et al., 1991; Tomkinson and Kieff, 1992; Tomkinson et al., 1993; Kaye et al., 1993; Roth et al., 1994; Khanna et al., 1997). In many respects, EBV immortalized cells look and behave like B cells that are stimulated to proliferate by incubation with CD40L and interleukin 4 (Gordon et al., 1986). In addition to CD19 and CD20 cell surface markers, infected proliferating B cells express CD23, CD30, CD39, CD70, CD80, CD86, CD58, and CD54. The density and serum dependence are suggestive of autocrine feedback loops operating to sustain and enhance growth (Gordon et al., 1984). All cells in a cultured cell line express the viral immortalization proteins and retain multiple unintegrated copies of the viral genome as episomes (Sugden and Mark, 1977; Sugden et al., 1979). Cell lines made by virus immortalization can be induced to differentiate toward a plasmacytoid phenotype by chemical or physical agents that trigger the latent episomes into a cell-lytic, virion-productive phase.

In addition to the protein-encoding genes, EBV was the first virus discovered to contain and express miRNAs (Pfeffer et al., 2004). Five miRNAs were initially found, clustered into two genomic regions. The miRNAs are expressed in all forms of B-cell latency, and the predicted silencing targets include B-cell-specific chemokines and cytokines and key regulators of transcription, signal transduction, cell proliferation, and apoptosis. Recently, 18 more miRNAs, 14 of which map to the introns of the BART gene, have been added to the list (Cai et al., 2006; Grundhoff et al., 2006). The EBV BART miRNAs accumulate specifically during latency and are evolutionarily conserved within the rhesus lymphocryptovirus genome, suggesting important roles for

these products in gammaherpesvirus infections. The pattern of EBV gene expression displayed by immortalized cells in culture has been termed latency III to distinguish this state from that observed in situ in the infected cells of EBV-associated diseases and circulating latently infected B cells. Three main categories of latent-type infections based on expression of these "latency-associated genes" are recognized: latency I (EBNA1 expression only), latency II (EBNA1, LMP1, and LMP2 expression only), and latency III (expression of EBNA1, LMP1, LMP2, and the other genes mentioned above). There is also a latency 0, characteristic of latently infected cells in the circulation of healthy carriers, where none of the genes are expressed.

The existence of latently infected cells in the blood and lymphoid tissues has been known for decades principally because explanted tissues rich in B lymphocytes spontaneously produced lymphoblastoid cell lines with the features of EBV-immortalized B cells (Pope et al., 1968; Nilsson et al., 1971; Rocchi et al., 1977; Rickinson and Epstein, 1985; Yao, 1985; Lewin et al., 1987). That a latent infection occurs in the lymphoid compartment was originally demonstrated by two key pieces of evidence. (i) During prolonged ACV treatment, virus shedding in the oropharynx ceased; however, the frequency of establishment of spontaneous immortalized B cell lines from the peripheral blood remained unchanged. (ii) Analyses of EBV strains recovered from bone marrow transplant recipients indicated that an existing latent infection was eradicated from seropositive recipients during replacement of their haematopoetic cell lineages with bone marrow from seronegative donors (Gratama et al., 1988; Yao et al., 1989). Consistent with these observations, individuals with X-linked (Bruton's) agammaglobulinemia, who produce no B lymphocytes and make no antibodies, have no detectable T-cell-mediated immune response to EBV antigens and no history of mononucleosis or evidence of EBV infection (Faulkner et al., 1999; Vihinen et al., 2000).

The presence and distribution of EBV-positive cells in lymphoid tissues has been examined by in situ hybridization with EBER probes (Howe and Steitz, 1986; Weiss and Movahed, 1989; Karlin et al., 1990; Strickler et al., 1993; Anagnostopoulos et al., 1995; Reynolds et al., 1995; Niedobitek et al., 1997a; Niedobitek et al., 1997b). In one study, 4 of 12 normal lymph nodes contained small EBER-positive cells localized to extrafollicular areas at a frequency of approximately 1/0.5 cm^2 to 10/0.5 cm^2 (Niedobitek et al., 1997a). This was contrasted with acute-stage mononucleosis lymph nodes where the distribution was similar but the frequency was >1,000/0.5 cm^2 and large positive cells were mixed in with the small positive cells. At the same time, the peripheral blood fills up with latently infected, resting memory B cells occupying as much as 50% of the peripheral memory compartment (Hochberg et al., 2004). During convalescence, this pool diminishes but never disappears. Using PCR to detect viral DNA in tissue specimens obtained at autopsy from patients with no indication of EBV-related disease, viral genomes were readily detected in the parotid gland (7/15), submandibular gland (8/10), nasopharynx (8/10), tonsil (8/10), larynx (5/6), lung (5/9), cervical lymph node (7/10), mediastinal lymph node (7/10), abdominal lymph node (4/10), spleen (6/10), and kidney (4/10) (Cheung and Dosch, 1991). Less-frequent detection was reported for the liver (1/10), pancreas (1/4), ovary (1/5), uterine cervix (1/4), and testis (1/3). Detection and quantitation of latently infected B cells in the peripheral blood of healthy carriers also has been studied (Wagner et al., 1992; Tierney et al., 1994; Miyashita et al., 1995; Miyashita et al., 1997). The frequency of EBV-positive cells in nine carriers ranged from $23/10^7$ to $625/10^7$ B cells with a mean of 125 (Miyashita et al., 1997). For a given individual, the viral load was relatively stable over a period of at least 2 years. By presorting peripheral blood lymphocytes based upon cell surface markers, infected cells were detected amongst the $CD19^+$, IgD^-, $CD23^-$, $CD80^-$, and $Ki67^-$ population, indicating that in situ latency is in nonactivated, nonproliferating, memory-type B cells (Miyashita et al., 1997; Babcock et al., 1998). Interestingly, the frequency of spontaneous outgrowth of lymphoblastoid cell lines is fivefold lower than the PCR-estimated number of latently infected cells (Babcock et al., 1998). This suggests that in vitro reactivation from latency may not be a very efficient process and/or that the in situ latently infected population may contain a large fraction of cells incapable of reactivating.

Epidemiology

The virus is transmitted primarily in the saliva, and early studies demonstrated the presence of biologically active virus in the salivary secretions of 15 to 20% of normal asymptomatic carriers (Gerber et al., 1972; Chang et al., 1973; Strauch et al., 1987). Examples of transmission through blood transfusion and along with the donor organ during transplantation have been documented, although these are considered to be atypical routes of transmission. Age of acquisition varies depending upon geographic location, being much earlier in resource-poor nations (>90% of preschoolers) than in Western Europe and America (30 to 40% of pre-schoolers) Socioeconomic status within developed nations also seems to influence transmission, as a greater percentage of poor children are seropositive in age-matched studies (Evans, 1982).

Two strains of EBV have been recognized (EBV-1 and EBV-2), and seroepidemiologic and virus isolation studies suggest that most people in Europe and North America are infected with the EBV-1 strain (Frezzera et al., 1981; Yao et al., 1991; Rickinson and Kieff, 1996). A much smaller percentage (<10%) are infected with the EBV-2 virus. Detection of double infections using discriminatory PCR assays on oropharyngeal secretions suggest that 10% of normal donors shed both types of viral DNA (Sixbey et al., 1989). Infections occur even in remotely isolated tribes in the Amazon basin and New Guinea highlands. Viruses recovered from these sites show nucleic acid polymorphisms that genetically distance them from both EBV-1 and EBV-2, suggesting that the virus can persist and evolve along with even very small groups of humans (Lung et al., 1990; Lung et al., 1991; Abdul-Hamid et al., 1992).

IM is not a reportable disease, so incidence statistics are not maintained. In one study, the reported rate in Connecticut was 48 cases/10^5 people/year for the general population, while a rate of 840 cases/10^5 people/year was recorded among college students, the group at greatest risk (Miller, 1990). No true epidemics of EBV infection have been described, although occasional clusters of cases have been reported.

Clinical Manifestations

IM

Most infections with EBV occur in childhood and are clinically unapparent (Miller, 1990; Rickinson and Kieff, 1996). Symptomatic infection in adolescents and adults is infrequent but can cause a recognizable clinical entity (IM). A recent study by Grotto and coworkers provides the most up-to-date description of the clinical and laboratory presentation of IM

in young adults (Grotto et al., 2003). The incubation period is between 30 to 50 days, followed by sore throat, headache, malaise, and fatigue. Fever lasts about 10 days. General lymphadenopathy involving the cervical, axillary, epitrochlear, inguinal, mediastinal, and mesenteric nodes is observed. Splenomegaly occurs in approximately 50% of cases and hepatosplenomegaly in approximately 10% of cases, but heart and/or CNS involvement is rare, and in immunocompetent patients, IM is rarely, if ever, fatal. Since EBV replication can be detected at high rates in the oropharynx during the symptomatic phase, trials with oral ACV have been conducted (Anagnostopoulos et al., 1995). While the drug did block virus shedding in the oropharynx, it did not alter the disease course or change the load of virus-infected cells in the blood (Yao et al., 1989; Anagnostopoulos et al., 1995). This observation has suggested that the disease symptoms are not caused by virus replication. An immunopathological etiology for the symptoms is suspected because they are precisely correlated with the period of atypical lymphocytosis caused by the exaggerated T-cell response and with the heavy infiltration of affected organs with these reactive T cells. The final consequence of EBV infection is a lifelong carrier state characterized by episodic asymptomatic shedding of virus into saliva, persistent low IgG antibody titers to EBNA1, a high frequency of circulating CTL precursors to EBV-infected B cells, and a corps of latently infected B cells that escape immunosurveillence (Miller, 1990; Rickinson and Kieff, 1996).

Lymphoproliferative Disease

EBV infection in the immunocompromised host is accompanied by the risk of developing lymphoproliferative disease. Posttransplant lymphoproliferative diseases (PT-LPDs) occur in approximately 1% of renal (Nalesnik et al., 1988; Wilkinson et al., 1989; Preiksaitis et al., 1992; Cockfield et al., 1993; Opelz and Henderson, 1993; Leblond et al., 1995), 2% of liver (Nalesnik et al., 1988; Wilkinson et al., 1989; Opelz and Henderson, 1993; Morgan and Superina, 1994), 6% of pancreas (Stratta et al., 1994; Davis et al., 1995; Lumbreras et al., 1995), 2 to 10% of heart (Nalesnik et al., 1988; Wilkinson et al., 1989; Swinnen et al., 1990; Preiksaitis et al., 1992; Opelz and Henderson, 1993; Chen et al., 1993; Leblond et al., 1995), 4 to 10% of heart-lung and lung (Nalesnik et al., 1988; Armitage et al., 1991; Preiksaitis et al., 1992; Leblond et al., 1995; Aris et al., 1996; Wood et al., 1996), 20% of small bowel (Kocoshis, 1994), 23% of multivisceral, and 2% of bone marrow transplants (Deeg et al., 1984; Kocoshis, 1994; Todo et al., 1995). Recent estimates indicate that 87% of PT-LPDs are of B-cell origin, 12.5% are of T-cell origin, and 0.5% are of null cell origin. For the B-cell PT-LPDs, the clinical and pathological features vary across a spectrum from polyclonal B-cell hyperplasia to monoclonal immunoblastic lymphoma (Nalesnik, 1998). The current view is that the disease is progressive (Tsao, 2007). Early stages have the characteristics of nonspecific reactive hyperplasia but can quickly evolve into a more diffuse polymorphic hyperplasia and finally into a disease with lesions resembling immunoblastic lymphoma or multiple myeloma (Frezzera et al., 1981; Nalesnik et al., 1988; Knowles et al., 1995; Harris et al., 1997). Since different lesions within the same patient may simultaneously present the features of different stages of disease, determining stage by inspection of tissue biopsy specimens is unreliable, and this has led to an inability to accurately predict clinical response to therapy based on histopathological assessment. HIV-infected individuals are also at risk for developing LPDs which morphologically have been divided into two broad categories, the BL-like lymphomas and the immunoblastic lymphomas (Hamilton-Dutoit et al., 1991). The BL-like lymphomas generally appear early in the progression of HIV disease toward AIDS, while the immunoblastic lymphomas are more likely to be diagnosed during the severely immuocompromised end stages of AIDS. These latter tumors are similar in most respects to the LPDs in transplant recipients, with the exception that primary CNS presentation (rare in transplant recipients) is common in AIDS lymphomas. It is the loss of EBV-specific CD4 T-cell help for CD8 T-cell responses that is primarily responsible for allowing EBV-associated lymphoproliferations to occur (Epeldegui et al., 2006). Over the last decade, the incidence of AIDS-associated lymphomas (and other cancers) has been declining among HIV carriers and the decline coincides with improving CD4 counts seen after the introduction of HAART in 1996 (Gingues and Gill, 2006; Valencia, 2006; Biggar et al., 2007).

OHL

Another disease manifestation of EBV infection that is a consequence of immunosuppression is oral hairy leukoplakia (OHL). It is characterized by a thickening of the epithelium at the lateral borders of the tongue (Greenspan et al., 1985). Histopathologically, there is a greatly disturbed basal to apical organization of the squamous epithelial layers that correlates with the presence of EBV in the affected cells. The lytic cycle marker BZLF1 is present, but expression is restricted to the apical regions of the lesion. This suggests that the lesion is an exaggeration of the normal course of virus shedding. The virus infects and is maintained in the basal self-renewing epithelial cells, replicates in the terminally differentiated cells, and is released in a manner analogous to the replication of papillomaviruses (Sixbey et al., 1984). While there has been a decrease in the prevalence of OHL, it was still the second-most common oral lesion in studies examining oral lesion prevalence in the decade after widespread use of HAART (Greenspan et al., 2004; Baccaglini et al., 2007). Because OHL is primarily a lytic virus lesion, it responds well to antiviral therapy (Resnick et al., 1988; Greenspan et al., 1990). Systemic valacyclovir and ACV are effective in approximately 90% of cases, but recurrence after cessation of therapy was between 20 and 100% (Resnick et al., 1988; Walling et al., 2003).

Virus-Associated Malignancies

The endemic African form of BL described by Dennis Burkitt shows a nearly 100% correlation with EBV. All the cells of the tumor are virus DNA and EBNA1 positive (Neri et al., 1991). Histologically, all of these tumors are dominated by a homogeneous population of small, noncleaved malignant B cells interspersed with occasional macrophages that give the specimen its characteristic "starry sky" appearance (Magrath, 1990). The nonendemic form of BL has only a 30 to 40% association with EBV. The current view of the pathogenesis of BL is that it is a multistep process involving environmental factors (that enhance germinal center activity), genetic alterations (the deregulation of the c-*myc* oncogene through an 8:14 chromosome translocation), EBV infection, and other factors and events not as well characterized. Thus, EBV infection contributes to but does not cause BL (Rickinson and Kieff, 1996).

The other well-documented EBV-associated tumor is nasopharyngeal carcinoma (NPC). The link between EBV and NPC was first suggested by elevated anti-EBV antibody

titers in NPC patients (Old et al., 1966). The presence of virus DNA in all cases of WHO type II/III undifferentiated NPC, and the monoclonal nature of the EBV episome in each case, has suggested a strong causal association (Raab-Traub and Flynn, 1986). Although NPC is rare in most populations, it is a leading form of cancer in southern China, Southeast Asia, the Arctic, and the Middle East/North Africa. This racial/ethnic and geographic distribution suggests that genetic traits combine with environmental factors in a multistep process in which the virus is just one player (Chang and Adami, 2006). The exact mechanism by which the virus contributes to NPC is not known.

Hodgkin lymphoma (HL) is a major lymphoma subtype in the Western world, accounting for nearly one-third of all lymphomas (Nakatsuka and Aozasab, 2006). Four WHO subtypes (nodular sclerosis, mixed cellularity, lymphocyte depleted, lymphocyte rich) and nodular lymphocyte predominant types are based on the characteristics of the reactive infiltrates and the morphology of Hodgkin/Reed-Sternberg (H/RS) tumor cell. A direct role for EBV in the development of HL has been proposed. In situ hybridization and immunohistochemical analyses have demonstrated that EBV can be found in H/RS cells in half of all HLs. For the two most common HL subtypes, EBV is more likely to be associated with the mixed cellularity subtype (70% of cases) than with the nodular sclerosis subtype (20% of cases). The role of EBV gene expression, which is restricted to EBNA1, LMP1, and LMP2, may be to regulate B-cell-specific gene transcription patterns that promote and maintain the H/RS cell phenotype (Nishikori and Uchiyama, 2006). The prevailing notion is that H/RS cells attract lymphocytes with T regulatory cell phenotypes and, through the release of anti-inflamatory cytokines, induce a profoundly immunosuppressive microenvironment that serves to protect the tumor cells.

Diagnosis

The clinical differential diagnosis of IM is difficult, especially in a complicated case or before the full-blown syndrome is evident. Mononucleosis must be distinguished from a variety of other infectious diseases that cause fever and inflammation of the upper respiratory tract. Serologic tests for the presence of antibodies to EBV are the principal means of confirming a diagnosis. The seroepidemiology of EBV infection was accomplished with a series of fluorescence assays developed during the first decade of research on the virus (Henle et al., 1968a, 1968b; Henle et al., 1970; Reedman and Klein, 1973). These measure three classical antigens: EBNA, EA (early antigen), and VCA (viral capsid antigen). A number of ELISA-based assays and other easy-to-read antibody tests based on specific peptide epitopes have been introduced (Pearson and Luka, 1986; Henle et al., 1987; Rowe et al., 1987; Hille et al., 1993; van Grunsven et al., 1994; Rowe and Clarke, 1998). The diagnosis of a primary EBV infection is based upon a test for EBV-specific IgG and IgM antibodies to VCAs and IgG antibodies to the EBNAs, especially EBNA-1, as the minimal requirement.

The diagram in Fig. 1 summarizes the kinetics of appearance and persistence of antibodies to EBV following primary infection of an immunocompetent host. IgM antibodies to VCA increase very early after infection and are usually substantial by the time symptoms appear. IgG anti-EA and IgG anti-VCA titers peak during the acute phase of IM disease, lagging slightly behind the atypical lymphocytosis but correlating well with the period of maximum virus shedding. The anti-EA responses drop faster and become undetectable

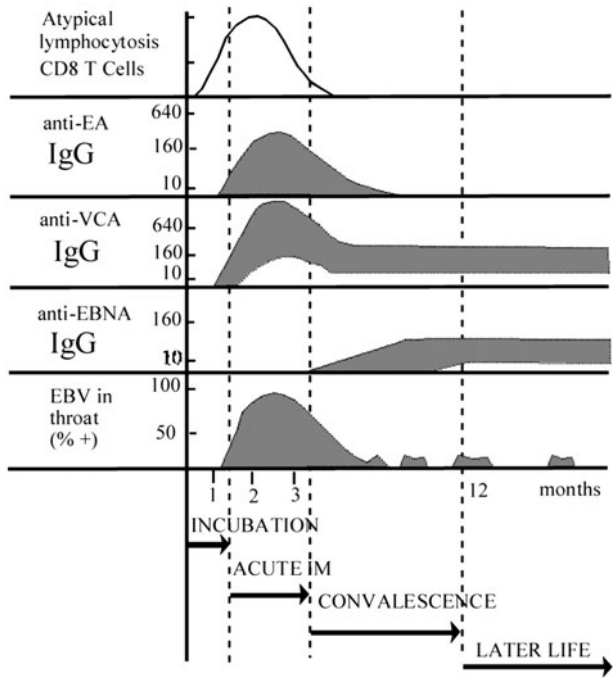

FIGURE 1 Antibody responses to EBV antigens during the course of IM are plotted relative to the period of atypical lymphocytosis and virus shedding. Reciprocal titers are shown for the virus-specific IgG class of antibody detected by immunofluorescence assays for the classical EA, VCA, and EBNA.

during convalescence. Persistently elevated anti-EA responses have been interpreted as evidence of active virus infection. The appearance of an anti-EBNA response is delayed and usually occurs late in infection during the convalescent phase. Patients with severe chronic active EBV infection, fatal mononucleosis, or a disseminated polyclonal lymphoproliferative disease often fail to develop any detectable anti-EBNA response (Henle and Henle, 1979; Rickinson and Kieff, 1996).

In addition to EBV-specific antibody responses, IM patients develop a nonspecific rise in the levels of serum immunoglobulins and the appearance of so-called heterophile antibodies, mostly of the IgM class, with autoreactive specificities. These antibodies appear very early in the course of infection and may be the products of virus-infected cells. They have the property of being able to agglutinate or lyse sheep, goat, horse, and bovine red blood cells and form the basis of the Paul-Bunnell or "spot" test for IM. The test may not be positive at first, may have to be repeated after 3 to 4 weeks, and may remain negative in young children even as EBV-specific serology confirms an infection is due to EBV.

The definitive diagnosis of an EBV-associated LPD requires histological examination of a tissue biopsy specimen and in situ hybridization with EBV-specific EBERs. RNA probes unambiguously define the involvement of virus-infected cells (Nalesnik, 1998). Because of the immunosuppressed state of the patient and the use of prophylactic pooled immunoglobulin therapy, EBV serology is unreliable. Because EBV viral loads in the peripheral blood are elevated at the time of a diagnosis of PT-LPD, EBV DNA load monitoring is a promising tool for the identification of patients at risk for PT-LPD development (Bakker et al., 2007). When measured

by quantitative PCR assays, there is consistently a difference of 3 to 4 orders of magnitude between the normal latent viral load of a healthy carrier and the load detected in a PT-LPD patient (Rowe et al., 1997). Monitoring viral load in the peripheral blood by quantitative PCR provides early detection of infection, an opportunity for preemptive therapeutic intervention, and a means of gauging the effectiveness of therapeutic strategies aimed at treating EBV infections and LPDs (Green et al., 1998; McDiarmid et al., 1999; Rowe et al., 2001; Lee et al., 2005).

Therapy

For IM, the use of the antiviral compounds ACV and GCV, as discussed above, is not effective. Therapy is essentially palliative. Bed rest is helpful and may be necessary in severe cases. Analgesics and antipyretics can provide relief from the sore throat pain and fever. Patients with enlarged spleens should avoid contact sports for 3 to 6 months following recovery. Approximately 30% of IM patients experience a secondary pharyngeal infection with group A streptococci that may require antibiotic treatment. Ampicillin is not recommended because it usually causes a rash in IM patients (Miller, 1990).

For LPDs, the picture is more complex. The disease is actually being caused by the underlying immunosuppression (Lim et al., 2006). If the immunosuppression can be reduced, for instance in PT-LPD in transplant recipients, then recovering T-cell immunity can eliminate the virus-driven LPD. This approach causes tumor regression in up to 50% of cases, usually within 4 weeks, and is most effective in the polymorphic forms of LPD (Paya et al., 1999). Preemptive reduction of immunosuppression in patients with high EBV viral loads, and therefore at high risk for PT-LPD, reduced the incidence of PT-LPD from 16 to 2% with a very low incidence of allograft rejection (Lee et al., 2005). A number of clinical studies have reported that transplant recipients given antiviral agents, principally as prophylaxis for posttransplant CMV infection, have had fewer PT-LPDs than expected from historical experience with similar recipients (Stratta et al., 1992; Davis et al., 1995; Lumbreras et al., 1995; Winston et al., 1995; Badley et al., 1997; Darenkov et al., 1997; Gane et al., 1997; Keay et al., 1998). This body of evidence has been balanced by an equal number of reports showing that prophylactic antiviral therapy has not prevented PT-LPD in nonrandomized trials (Shapiro et al., 1988; Zutter et al., 1988; Walker et al., 1995; Aris et al., 1996; Wood et al., 1996; Haque et al., 1996; Newell et al., 1996). Some of the studies have supplemented antivirals with various doses of intravenous immunoglobulin, while others have not included any immunoglobulin. The value of supplemental immunoglobulin in prophylaxis for EBV infections has not been determined.

Rituximab (Rituxan, Mabthera), a chimeric monoclonal antibody recognizing the CD20 antigen on the surface of mature B-cell lymphocytes, has been used increasingly in the treatment of PT-LPD. Rituximab was initially approved for the treatment of low-grade non-HLs, but multiple case studies, retrospective analyses, and phase II trials demonstrate the benefit of rituximab in PT-LPD (Svoboda et al., 2006). Long-term complete remissions have been reported in 30 to 70% of PT-LPD patients after a single rituximab treatment (Webber et al., 2004; Oertel et al., 2005; Svoboda et al., 2006; Choquet et al., 2006). The different range of experience appears to depend upon whether (and how many) patients with fulminant, advanced disease and EBV-negative or late-onset tumors (all of whom are less likely to respond to single-agent rituximab) are included in the treatment evaluation (Svoboda et al., 2006).

Adoptive transfer of donor-derived EBV-specific CTLs to allogeneic bone marrow transplant recipients have been tried (Heslop et al., 1994; Papadopoulos et al., 1994; Rooney et al., 1995 Heslop et al., 1996; Bonini et al., 1997). In one series, five patients with PT-LPD were treated with infusions of donor peripheral blood mononuclear cells achieving complete and sustained remissions (Papadopoulos et al., 1994; Heslop et al., 2004).

Using CTL therapy in combination with other treatment modalities (e.g., chemotherapy or rituximab) for aggressive PT-LPDs that are unresponsive to reduced immunosuppression has successfully induced remission without significant side effects (Comoli et al., 2005). One of the hurdles facing adoptive immunotherapy is the timely production of infusible autologous CTLs. The delay can be on the order of 4 to 6 months, which is too long for patients with active disease. In addition, the feasibility of establishing CTLs from previously seronegative transplant recipients presents its own unique set of challenges (Comoli et al., 2005). Improved strategies for growing EBV-specific CTLs in vitro are still in development but will soon permit expanded trials in seronegative as well as seropositive solid-organ transplant recipients and other immunocompromised patients (Popescu et al., 2003; Comoli et al., 2005; Popescu et al., 2007). One alternative and promising strategy currently being investigated is the use of pregenerated cryopreserved CTLs from a "bank" of CTLs made from EBV-seropositive donors. In recent trials, partially HLA-matched, and therefore heterologous, CTLs were used to treat polyclonal hyperplastic PT-LPD and fulminant, advanced disease with some success and negligible toxicity (Haque et al., 2002; Gandhi et al., 2007). Larger prospective studies using this approach are warranted.

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Human Herpesviruses 6, 7, and 8

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Human herpesvirus 6 (HHV-6), HHV-7, and HHV-8 (also known as Kaposi's sarcoma [KS]-associated herpesvirus) were discovered between 1986 and 1994 (Salahuddin et al., 1986; Frenkel et al., 1990; Chang et al., 1994). These are not new viruses in an evolutionary sense; they have been part of human biology since near its inception. That they were discovered only recently is a measure of both their subtle interaction with immunocompetent hosts and the relative youth of virology. Their discoveries can be attributed to the conflux of increasing populations of immunocompromised patients and the development of methods for lymphocyte propagation and molecular identification of infectious agents.

Two forms of HHV-6 have been identified, variants A and B (HHV-6A and HHV-6B). HHV-6B is the major etiologic agent of roseola (also known as roseola infantum, exanthem subitum, or sixth disease); the disease spectrum of HHV-6A remains an enigma. HHV-7 accounts for a subset of roseola cases. Both of the HHV-6 variants and HHV-7 have been associated with disease in immunocompromised organ transplant recipients. HHV-8 is the etiologic agent of KS and has been closely associated with multicentric Castleman's disease (MCD) and a form of body cavity lymphoma known as primary effusion lymphoma (PEL). Each of the viruses also has been associated with a number of other diseases for which proof of an etiologic role is incomplete or has been refuted. Prevalences and disease associations are summarized in Tables 1 and 2.

Like other herpesviruses, these viruses establish lifelong infections in their host. These persistent infections are maintained through a combination of latent (nonproductive) infections in some cells and intermittent or persistent lytic infections in other cells or tissues. Latent virus can be reactivated to a lytic state from which it can reseed the latent and persistent repositories and provide infectious material for transmission. Reactivation events are normally subclinical but can be associated with disease, particularly in immunocompromised patients.

These viruses present diagnostic challenges for the clinician and the laboratorian. It is likely that much will change on these fronts over the next few years, with further development of both rapid diagnosis and therapy that will work best in conjunction with careful monitoring of viral activity.

HHV-6

Discovery and Identification of the Variants

Salahuddin and colleagues discovered HHV-6 in lymphocytes of immunocompromised patients with lymphoproliferative disorders (Salahuddin et al., 1986). Isolates of the virus segregate into two distinct groups (HHV-6A and HHV-6B) based on their in vitro cell tropisms, antigenicity, nucleotide sequences, and epidemiology (reviewed in Braun et al., 1997; Campadelli-Fiume et al., 1999). While distinct, HHV-6A and HHV-6B are more closely related than any pair of recognized herpesvirus species. Many aspects of their biology overlap, and they are sufficiently antigenically cross-reactive that variant-specific serologic assays are not available. Nonetheless, because they are distinct, we will discuss them separately to the extent possible. When discussing studies where distinctions were not made, we will refer to them collectively as HHV-6.

Biology

Structure

HHV-6A and HHV-6B share the morphology that is common to all herpesviruses: an electron-dense core containing the viral genome that is contained in a capsid, which is surrounded by a proteinaceous layer known as the tegument, all of which is enclosed in a lipid envelope that is studded with virally encoded glycoproteins and integral membrane proteins. Virions are 150 to 200 nm in diameter and have a characteristic appearance by electron microscopy (Fig. 1A).

Genetic Content and Taxonomy

HHV-6 genomes are approximately 160 kb in length and encode approximately 100 genes (reviewed in Yamanishi et al., 2007). The genomic termini are bounded by arrays of the sequence element, (TAACCC)n; similar sequences are present at the telomeres of mammalian chromosomes. Different groups of genes are conserved at each taxonomic level, proceeding from the level of family to subfamily, genus, and species. About 40 genes are homologs of genes that are shared across the herpesvirus family. Some genes are represented in all members of the betaherpesvirus subfamily (including human cytomegalovirus [HCMV]); a handful are specific to the *Roseolovirus* genus (the HHV-6 variants and HHV-7).

TABLE 1 Prevalences of the recently discovered herpesviruses

Virus and area type	Prevalence (%)	
	Children (3 to 12 yr)	Adults
HHV-6A	?[a]	>50
HHV-6B	>80	>90
HHV-7	70	60–95
HHV-8		
KS nonendemicity	0–5	2–10
KS endemicity	10–30	30–>50

[a]?, unknown.

The U94 gene is common to HHV-6A and HHV-6B but not to HHV-7; U94 is a homolog of the gene encoding the important parvovirus nonstructural protein, *ns*1 or *rep*. Between the variants, amino acid sequence identities generally range from 94 to 99% in the genes that are conserved across the herpesvirus family and 70 to 90% in other genes. Several genes are specific to each of the HHV-6 variants.

Taxonomy

As described above, HHV-6A and HHV-6B are classified in the *Roseolovirus* genus of the betaherpesvirus subfamily; the variants have not been formally recognized as distinct species (Braun et al., 1997; Campadelli-Fiume et al., 1999; Dominguez et al., 1999).

Growth Cycle

The HHV-6 variants can infect a variety of cell types in vitro but grow best in primary umbilical cord blood lymphocytes. Several strains have been adapted for reliable replication in continuous lymphocyte cell lines. HHV-6A isolates grow in HSB-2 and JJHAN cells and HHV-6B variants grow best in Molt-3 and MT-4 cells. Pathologic studies indicate that the in vivo host cell range is much wider and includes cells of lymphoid, epithelial, and neuronal origin (Table 2) (reviewed in Braun et al., 1997). CD4-positive cells are frequent targets of infection, although CD4 itself is not the receptor. HHV-6-infected lymphocytes become greatly enlarged and refractile in 3 to 5 days. Some cells become multinucleated. Host cell protein synthesis continues in the face of infection, although host DNA replication is shut down. In general, infectious yields are low, with titers normally ranging from 10^3 to 10^5/ml.

Cells of the myeloblast lineage are important in vivo. Thus, polymorphonuclear leukocytes (PMNL) harbor more viral DNA per cell than do peripheral blood mononuclear cells (PBMC) from the same individual, and both HHV-6A and HHV-6B replicate better in PMNL than in PBMC (Palleau et al., 2006). Similarly, cells of the monocyte/macrophage lineage harbor severalfold-more HHV-6 than PBMC during acute HHV-6 infection (Kondo et al., 2002).

The major cell surface receptor for HHV-6A and HHV-6B is CD46; the virus may use an additional receptor(s) (Pedersen and Hollsberg, 2006). HHV-6 DNA replicates in the nucleus and is initiated by a process more akin to alphaherpesviruses than to other betaherpesviruses. HHV-6 matures along a pathway that includes (i) envelopment of tegumented capsids at inner nuclear membranes that lack the full complement of viral glycoproteins; (ii) de-envelopment into the cytoplasm; (iii) reenvelopment at annulate lamellae or *cis*-Golgi cisternae membranes that are studded with viral glycoproteins; (iv) progressive maturation of virion glycoproteins during passage through the Golgi; and (v) virion release from vesicles at the cell surface or during cell lysis (Yamanishi et al., 2007).

Epidemiology

Due to the lack of variant-specific serologic assays, it is difficult to describe the distribution and prevalence of the variants individually. The combined seroprevalence exceeds 90% and can approach 100% (Table 1) (reviewed in Braun et al., 1997). HHV-6B infections occur most often before the age of 2; nearly everyone who is HHV-6 seropositive is likely to have been infected with this variant. HHV-6A prevalence is more difficult to pin down, but PCR studies of lung and skin biopsy specimens suggest that it may exceed 50% in the United States and Italy (Cone et al., 1996; Di Luca et al., 1996). The age of acquisition for HHV-6A has not been identified.

Transmission

Saliva is the probable primary route of transmission for HHV-6B (Mukai et al., 1994; Di Luca et al., 1995; Tanaka-Taya et al., 1996; Rhoads et al., 2007). Viral DNA has been detected in varying percentages of cervical secretions, cord blood, and tissues from spontaneously aborted fetuses (reviewed in Braun et al., 1997), suggesting that congenital or sexual transmission is possible. Consistent with this, 5 (7%) of 72 patients in a neonatal intensive care unit who were tested within 7 days of birth had HHV-6 DNA in their plasma (Rentz et al., 2007). HHV-6A has been detected but more commonly in skin than saliva (Di Luca et al., 1996), suggesting that the virus might be transmitted by direct contact.

HHV-6 can also be transmitted genetically via genomes that are integrated near the telomeres of chromosomes of germ line cells (Torelli et al., 1995; Daibata et al., 1998; Morris et al., 1999). Chromosomally integrated HHV-6 (ciHHV-6)

TABLE 2 Tissue distribution of and diseases associated with HHV-6A, HHV-6B, HHV-7, and HHV-8

Virus	Predominant tissue tropism in vivo	Principal clinical syndrome(s)
HHV-6A	$CD4^+$ lymphocytes, skin, brain, monocytes, lung	AIDS- and transplant-associated opportunistic infection
HHV-6B	$CD4^+$ lymphocytes, PMNL, monocytes, salivary glands, lymph node, brain, lung	Febrile illness, roseola, posttransplant disease
HHV-7	$CD4^+$ lymphocytes, salivary glands	Subset of roseola, CMV-associated posttransplant disease
HHV-8	Diseased tissue associated with KS, PEL, MCD; $CD19^+$ PBMC	KS, MCD, PEL

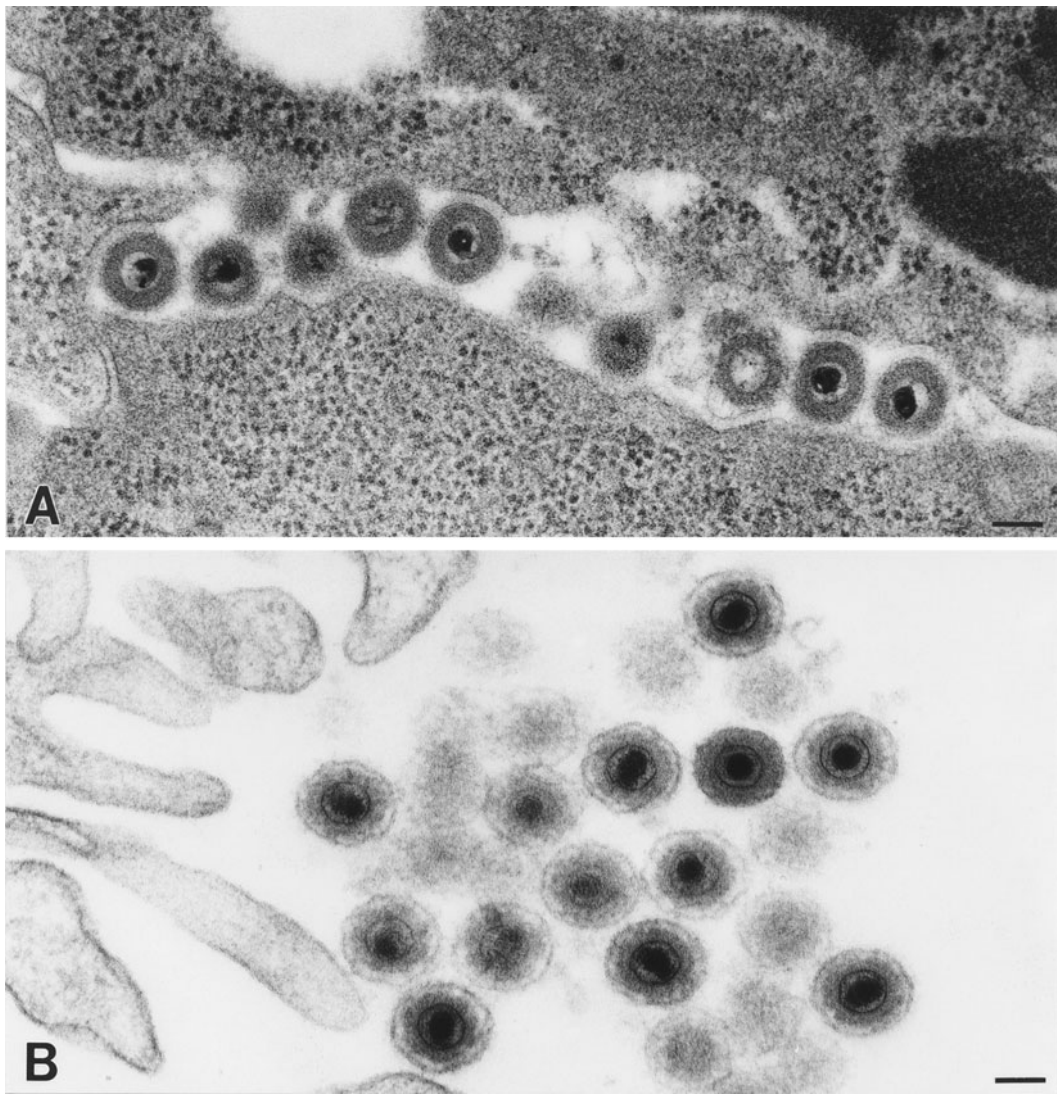

FIGURE 1 HHV-6B (A) and HHV-7 (B) virions grown in cord blood lymphocytes. Virus particles are extracellular and mature. Culture material was provided by Jodi Black and Carlos Lopez, and electron microscopy and photography were by Cynthia Goldsmith, Centers for Disease Control and Prevention. Bars = 100 nm.

has been detected in 0.21% to 5% of individuals in various populations (Tanaka-Taya et al., 2004; Leong et al., 2007; Ward et al., 2007). Transplantation of stem cells from individuals with ciHHV-6 can result in populating the recipient's lymphoid compartment with cells that harbor the viral genome (Clark et al., 2006a). Chromosomal integration leads to very high levels of viral DNA in cells (at least one or two viral genomes per cell) and in fluids such as plasma and cerebrospinal fluid (CSF). This needs to be considered in evaluating the possible role of the virus in a given clinical circumstance. In some instances of very high levels of HHV-6 DNA in PBMC, the viral genome did not appear to be present in every cell (Luppi et al., 2006), possibly due to (i) intrauterine transmission early during gestation, so that the viral genome is integrated into only a subset of cells or cellular lineages; (ii) germ line chromosomal transmission with subsequent loss of integrated genomes from a subset of cells or cellular lineages; or (iii) insufficiently sensitive methods for detecting integrated genomes (Clark et al., 2006b; Luppi et al., 2006; Pellett and Goldfarb, 2007).

Pathogenicity and Clinical Aspects

The significant clinical aspects of HHV-6A and HHV-6B infections can be broken down by age (children and adults) and immune status. Further major categories include primary infections or reactivated lytic infection and the possibility that latent infection can have clinical consequences. Disease associated with primary infection normally occurs early in life, while reactivated latent virus (or very rare primary infections) is associated with disease in immunocompromised organ transplant recipients. Numerous diseases have been associated with HHV-6 infections (Table 3). For roseola, the evidence is robust and incontrovertible. For some other diseases, such as following organ transplantation, the available

TABLE 3 Spectrum of HHV-6 disease associations

Age group	Associated disease	
	Proven or highly probable	Proposed[a]
Children	Roseola Febrile illness Febrile convulsions Meningitis and encephalitis Posttransplant disease	Hepatosplenomegaly Thrombocytopenia
Adults	Lymphadenopathy Mononucleosis-like illness Meningitis and encephalitis Posttransplant disease	DIHS Acute lymphoblastic leukemia MS Non-Hodgkin's lymphoma S100$^+$ chronic lymphoproliferative disease Spontaneous abortion

[a]Etiology not proven because data are either lacking or conflicting.

data are compelling and consistent with a pathogenic role. Multiple sclerosis (MS) is a disease for which intriguing and tantalizing observations of a possible etiologic association have not always been consistent but for which evidence continues to accumulate that the virus is more than an occasionally detected bystander. In some cases, proof of an etiologic association is difficult to come by because of the scarcity of a disease.

HHV-6 is a commensal inhabitant of the central nervous system (CNS), with over 80% of adult brains harboring virus (Luppi et al., 1994), and is typically present in a latent form in circulating lymphocytes, CD34$^+$ hematopoietic progenitor cells, and their differentiated progeny, including monocytes and macrophages (Kondo et al., 1991; Katsafanas et al., 1996; Luppi et al., 1999), and PBMC-derived dendritic cells and PMNL (Asada et al., 1999; Palleau et al., 2006) (Table 2). The only viral transcripts identified in latently infected lymphocytes include latency-specific spliced isoforms of the major immediate early gene (Kondo et al., 2003) and the U94 gene (Rotola et al., 1998).

The pathogenic effects of HHV-6 can be direct or indirect. By direct, we mean tissue damage due to viral replication or to localized responses to lytic viral replication. Indirect effects include the possibility that the virus has immune modulatory effects (reviewed in Ljungman and Singh, 2006; Wang and Pellett, 2006). Mechanisms of immune modulation include impairment of differentiation of monocytes to dendritic cells (Niiya et al., 2006) and delayed T-cell proliferative responses during primary infection (Kumagai et al., 2006). Relative to uninfected individuals, healthy adults with latent HHV-6 infections are tilted toward T regulatory type 1 cell activity, with interleukin-10 (IL-10)-linked selective impairment in their CD4 proliferative responses and reduced numbers of stimulated cells that express gamma interferon (Wang et al., 2006a).

Primary Infection

HHV-6B is an important pathogen in young children. Primary HHV-6B infections normally occur after maternal antibody wanes at around 6 months of age, but infections can occur earlier. Approximately 30% of children in the United States experience roseola, which is a normally mild, self-limiting disease characterized by 1 to 3 days of fever (39 to 40°C) followed by a rash that lasts 1 to 3 days. A transient immunoglobulin M (IgM) response develops quickly, followed within 2 weeks by a sustained IgG response of increasing antibody avidity (Balachandra et al., 1991; Ueda et al., 1989; Ward et al., 1993a) (Fig. 2). Natural killer cell activity is elevated early during primary infection and is probably important in controlling infection (Kumagai et al., 2006). HHV-6B can be detected by culture most efficiently during the febrile phase that precedes the rash in roseola (Asano et al., 1991a; Okada et al., 1993). Viral DNA can be readily detected in blood by PCR for over a month after primary infection (Pruksananonda et al., 1992).

Primary infection may also present as fever without rash or rash without fever. In a prospective study, symptoms were reported in 94% of children with primary infection, the most common being fussiness, rhinorrhea, cough, vomiting, diarrhea, and roseola (Zerr et al., 2005). Children can also experience more severe symptoms in association with primary HHV-6B infection, including fever of ≥39.5°C, inflamed tympanic membranes, gastrointestinal and respiratory distress, thrombocytopenia, transient acquired myelodysplasia, intussusception, and febrile seizures (Table 3) (Asano et al., 1991b; Pruksananonda et al., 1992; Hall et al., 1994; Yoshikawa et al., 1998). HHV-6 infections account for 20 to 40% of febrile admissions to pediatric emergency departments for children 6 months to 2 years old (Pruksananonda et al., 1992; Portolani et al., 1993; Hall et al., 1994; Kagialis-Girard et al., 2006). Papers from the Asano-Yoshikawa and Hall-Caserta groups are particularly informative on these issues and are highly recommended (Asano et al., 1991a.; Asano et al., 1994; Pruksananonda et al., 1992;

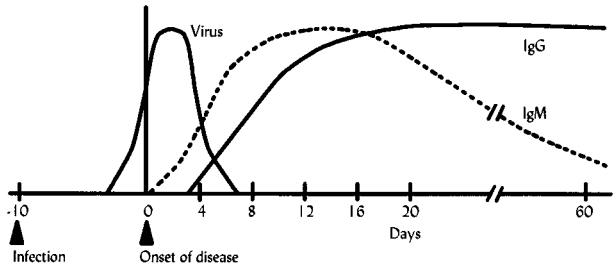

FIGURE 2 Immune response to HHV-6 showing the temporal relationship between viral load, the clinical signs and symptoms of infection (roseola), and the production of specific IgM and IgG antibodies.

Suga et al., 1993; Caserta et al., 1994; Caserta et al., 1998; Caserta et al., 2004; Hall et al., 1994; Hall et al., 2006).

Primary infections with HHV-6 and -7 most often occur at times coincident with or immediately following routine childhood immunization, leading to possible misdiagnosis of an adverse reaction to the vaccine (Ward et al., 2005). Some children with high fever due to primary HHV-6 infection are treated with antibiotics. Furthermore, a roseola rash may appear following antibiotic therapy, which could be mistakenly diagnosed as a drug allergy, unnecessarily precluding future use of the drug. A rapid, point of care, diagnostic assay for HHV-6 infection may be helpful in reducing unnecessary use of antibiotics and hospital admissions, but HHV-6 status alone is insufficient in determining whether to evaluate for serious bacterial infection (Zerr et al., 2006).

Disseminated, sometimes fatal, HHV-6 infections have been described in children as well as associations with cases of idiopathic thrombocytic purpura and hemophagocytic syndrome (reviewed in Braun et al., 1997; Hoang et al., 1999).

Primary infection in adults is rare but can be severe. It has been associated with lymphadenopathy, hepatosplenomegaly, and other symptoms similar to mononucleosis (reviewed in Braun et al., 1997; De Bolle et al., 2005).

Disease in Immunocompromised Patients

Because these viruses establish lifelong latent infections, viral activity in immunocompromised patients can occur without new viral exposure. During the posttransplant period, HHV-6 activity is detectable in about half of hematopoietic stem cell transplant (HSCT) recipients and solid-organ recipients, most commonly 2 to 4 weeks after transplantation. Most of the time, this activity is not associated with illness, but as detailed below, HHV-6 does cause mild to severe illness in some patients. The importance of HHV-6 monitoring is underscored by cases in which HHV-6-associated CNS symptoms were thought to be the product of tacrolimus- or cyclosporine-induced encephalopathy, leading to withdrawal of the immune-suppressive drugs and subsequent development of sometimes fatal graft-versus-host disease (GVHD) (Fujimaki et al., 2006). About 90% of the HHV-6 that has been detected following organ transplantation has been HHV-6B. Disease associations include febrile rash episodes that resemble GVHD, sinusitis, pneumonitis, graft suppression and rejection, encephalitis, and possibly increased risk of "CMV disease" (reviewed in Braun et al., 1997; De Bolle et al., 2005). While sometimes linked to GVHD, it is not clear whether viral activity is the cause of GVHD or is caused by GVHD or whether the infection or GVHD are sometimes misdiagnosed as the other.

The degree of HHV-6 activity and extent of association with disease in transplant recipients has varied from study to study. Major variables include recipient age, the organ being transplanted, immune-suppressive regimen and other aspects of case management, material assayed (circulating lymphocytes, tissue biopsy specimens, serum, or urine), assay methods (serology, quantitative versus qualitative PCR, or variant identification), interactions with other viruses (especially HCMV and HHV-7), and study design (e.g., intensively studied cohort or retrospective analysis of materials collected from patients who fit a particular profile, such as having idiopathic pneumonitis). Investigators at some centers have consistently charted a more benign course of posttransplant HHV-6 activity than have others (Carrigan and Knox, 1994; Cone et al., 1999).

For renal transplant recipients, HHV-6 activity has been most frequently observed in patients who received anti-lymphocyte therapy (anti-CD3 monoclonal antibody or anti-lymphocyte globulin) as part of their immunosuppressive regimen. This is possibly related to the enhancing effect of anti-CD3 antibodies on viral growth that has been observed in vitro (Kikuta et al., 1990; Roffman and Frenkel, 1991). HHV-6 antigens are much more likely to be detected in allografts during acute rejection than during chronic rejection. In a study where HHV-6B and not HHV-6A was detected in most categories of patients and materials, of 24 renal transplant recipients, HHV-6A was detected in PBMC from 9 and HHV-6B was detected in 15 (Boutolleau et al., 2006b). Interestingly, specific polymorphisms in the p53 coding region were associated with >5-fold increased risk for HHV-6 infection in renal transplant recipients (Leite et al., 2006). Specific outcomes associated with HHV-6 activity have included rejection, viral syndrome, CMV syndrome, elevated liver enzymes, and colitis (Okuno et al., 1990; DesJardin et al., 1998; Ratnamohan et al., 1998; Wade et al., 1998; Chapenko et al., 2001; Delbridge et al., 2006). Simultaneous detection of HCMV and HHV-6 in urine or serum was a strong predictor of severe disease (Ratnamohan et al., 1998).

In pediatric liver transplant recipients, 4 (12%) of 33 patients had HHV-6 DNA detected in their serum by PCR; primary infection was associated with moderate to severe acute graft rejection (Feldstein et al., 2003) and there were no associations between HHV-6 activity and HCMV or HHV-7. Adults who received liver transplants due to acute liver failure, and whose livers harbored HHV-6 antigens prior to receiving a transplant, were at higher risk for posttransplant HHV-6B infections of the liver and associated liver dysfunction, but there was no adverse effect on 1-year survival (Harma et al., 2006a). Patients with symptomatic CMV infections were likely to also have simultaneous HHV-6B and HHV-7 antigenemia (Harma et al., 2006b). HHV-6 activity has been associated with fungal infections in liver transplant recipients (Dockrell et al., 1999; Rogers et al., 2000).

In heart or heart-lung transplantation, HHV-6 antigenemia was detected in 20 (91%) of 22 recipients, including temporally linked cases of encephalitis and pneumonitis (Lehto et al., 2007). Detection of HHV-6 DNA in bronchoalveolar lavage fluid is a risk factor for acute rejection and development of relatively severe bronchiolitis obliterans syndrome and death (Neurohr et al., 2005).

In HSCT recipients, HHV-6 activity has been associated with encephalitis, sinusitis, GVHD and other exanthems, pneumonitis, idiopathic febrile episodes, marrow suppression, delayed granulocyte and platelet engraftment, and higher mortality (reviewed in Braun et al., 1997; De Bolle et al., 2005; Ljungman and Singh, 2006). Delayed engraftment also has been associated with high levels of viral DNA in plasma or antigenemia (Maeda et al., 1999; Savolainen et al., 2005; Ogata et al., 2006), including a specific association with detecting HHV-6B (but not HHV-6A) DNA in plasma before posttransplant day 28 (Radonic et al., 2005). Risk factors for HHV-6 DNAemia in HSCT recipients include donor-recipient HLA mismatches, conditioning with antithymocyte globulin, administration of steroids, and low HHV-6 antibody titers at the time of transplantation (Volin et al., 2004; Ogata et al., 2006; Wang et al., 2006b; Yamane et al., 2007). HHV-6A and HHV-6B antigenemia were detected in 33% and 62% of allogeneic stem cell recipients, respectively. No clinical events were associated with HHV-6A, but HHV-6B was associated with rash and GVHD (Volin et al., 2004). There was no evidence of additive or

synergistic pathogenic effects between HCMV, HHV-6, and HHV-7 in allogeneic stem cell recipients (Sassenscheidt et al., 2006), and although HHV-6 activity was detected in most HCMV-seronegative HSCT recipients, the associated illnesses were mild and self-limited (Cone et al., 1999). Likewise, among 60 pediatric stem cell recipients, HHV-6 activity was frequently detected, sometimes in association with transient illness, but severe complications were not observed, regardless of the graft being allogeneic or autologous (Savolainen et al., 2005).

Perhaps the most common severe consequence of HHV-6 infection in HSCT recipients is encephalitis, which occurs in about 1% of recipients and has a mortality rate of about 40% (Ljungman and Singh, 2006; Zerr, 2006). Symptoms include altered mental status, reduced consciousness, convulsions, amnesia, lethargy, and confusion. Onset is often between 2 and 4 weeks following the transplant and is most often associated with HHV-6B. Treatment with ganciclovir and/or foscarnet may be useful (Zerr et al., 2002), but there have not been controlled trials, and improvements have been observed in the absence of antiviral therapy (Sassenscheidt et al., 2006). Associations have been seen in cases where the conditioning regimen included anti-lymphocyte globulin or anti-CD3 (Zerr et al., 2002) or the anti-CD52 monoclonal antibody, alemtuzumab (Vu et al., 2007), and in patients who received HLA-mismatched stem cells (Fujimaki et al., 2006).

The virologic and clinical course of disease was particularly well described for three cases in which recipients of bone marrow stem cells died within weeks of HHV-6B-associated neurologic complications (Fotheringham et al., 2007a). Viral loads were elevated in CSF prior to their elevation in serum and peaked 2 to 4 weeks before death. Viral antigens were readily detected in brain tissues, concentrating in hippocampal astrocytes. High loads of viral DNA in brain tissues were seen at a time when levels in CSF were low, suggesting that detection of even low levels of viral DNA in CSF should alert the physician to possibly significant viral activity in the brain. In magnetic resonance imaging of cases of HHV-6-associated neurologic disease following stem cell transplantation, abnormalities were consistently seen in the mesial temporal lobe region, including the hippocampus/amygdala (Gorniak et al., 2006; Noguchi et al., 2006).

HHV-6 has been hypothesized to have a role in AIDS based on several in vitro studies. HHV-6 and human immunodeficiency virus (HIV) can up- or downregulate each other's growth (depending on conditions), HHV-6 gene products can induce expression from the HIV long terminal repeat, and HHV-6 can induce or upregulate CD4 expression, increasing the pool of cells susceptible to HIV infection (reviewed in Braun et al., 1997). Late in AIDS, HHV-6 DNA and antigens can become disseminated into many tissues. Evidence for in vivo synergism between HIV and HHV-6B includes elevation of loads of both viruses by approximately 1 $\log_{10}$ when both viruses are present in tissues (Emery et al., 1999). In a study of HHV-6 in children vertically infected with HIV (Kositanont et al., 1999), HHV-6B infection was acquired more slowly by the HIV-infected children (no HHV-6A infections were detected). Children 4 to 12 months old with low $CD4^+$ cell ratios (<15%) were less likely to acquire HHV-6, suggesting that the lower rate of HHV-6 acquisition was due to target cell depletion. Further, HIV disease progressed in all children who acquired HHV-6 infections but not in the HIV-infected children who remained free of HHV-6.

Immunity can also be compromised in cancer patients, due to either the cancer itself or to cytotoxic chemotherapy. Michalek and Horvath found primary and reactivated HHV-6 infections in about a third of pediatric cancer patients during their therapy; some of these events were associated with clinically significant disease, including pneumonitis, bone marrow aplasia, and persisting fever (Michalek and Horvath, 2002).

Disease in Immunocompetent Individuals

HHV-6 infections have been associated with numerous diseases in immune-competent individuals (Table 3) (reviewed in Braun et al., 1997; De Bolle et al., 2005; Dewhurst, 2004) for neurologic diseases. Several warrant mention here, including (i) viral myocarditis, (ii) drug-induced hypersensitivity syndrome (DIHS), (iii) encephalitis or encephalopathy, (iv) temporal medial lobe epilepsy, (v) MS, and (vi) other disease associations.

Viral Myocarditis

Viral infections of the myocardium can lead to cardiac dysfunction. HHV-6 has been detected in cardiac aortic endothelial cells (Rotola et al., 2000) and in endomyocardial biopsy specimens of patients with peripartum cardiomyopathy and controls from whom biopsy specimens were taken for other reasons (Bultmann et al., 2005). In cardiac tissues from women with peripartum cardiomyopathy but not in the controls, the virus was found in regions of marked interstitial inflammation. HHV-6 DNA was detected in 23% of biopsy specimens from patients with viral myocarditis, half with HHV-6 alone and half with HHV-6 plus parvovirus B19 (Kuhl et al., 2005). Patients who cleared the infections over subsequent months had better heart function than did patients with persistent infections. In another study, HHV-6 and combined HHV-6–parvovirus B19 infections were linked to new onset heart failure with septal late gadolinium enhancement being observed by magnetic resonance imaging (Mahrholdt et al., 2006); many of these cases progressed to chronic failure.

DIHS

DIHS, also known as either drug reaction with eosinophilia and systemic symptoms, or anticonvulsant hypersensitivity syndrome, is a severe multiorgan disease that occurs following exposure to some anticonvulsant drugs (allopurinol, carbamazepine, dapsone, gabapentin, mexiletine, minocycline, phenobarbital, phenytoin, and salazosulfapyridine) (reviewed in Matsuda et al., 2006; Oskay et al., 2006; Shiohara et al., 2006) and the industrial solvent trichloroethylene (Huang et al., 2006). HHV-6 activity is frequently observed in DIHS patients, with marked increases in antibody titers being seen approximately 3 to 4 weeks following onset, sometimes accompanied by PCR positivity. Viral activity follows the onset of DIHS, suggesting that the viral activity is a response to the disease, rather than being its cause, although it remains possible that activated virus may add to the disease burden. Shiohara et al. have proposed that elevation in HHV-6 serologic responses constitutes a gold standard marker for discriminating DIHS from other drug eruptions, with diagnosis being made on the presence of five of the following six criteria: maculopapular rash, lymphadenopathy, fever (>38°C), leukocytosis, hepatitis, and HHV-6 reactivation (Shiohara et al., 2006). The temporal progression of detecting HHV-6 and HHV-7 3 to 4 weeks after DIHS onset, followed by detection of HCMV and Epstein-Barr virus (EBV) activation 2 to 3 months later

has marked similarities to the progression of viral activity following organ transplantation (Seishima et al., 2006).

Encephalitis or Encephalopathy

While less common among immune-competent individuals, there have been a number of reports of HHV-6 associations with CNS diseases (McCullers et al., 1995; Kolski et al., 1998). It has been argued that in some cases of suspected HHV-6 encephalitis, the virus may have been incorrectly blamed for the disease, with the observed PCR positivity possibly having been due to the ease of detecting integrated viral genomes (Ward, 2005; Ward et al., 2006; Ward et al., 2007; Whitley and Lakeman, 2005). Clark, Ward, and colleagues make a strong case that diagnosis of suspected HHV-6 encephalitis should exclude the possibility of ciHHV-6, for example, by evaluating viral load over time and in CSF, serum, and/or whole blood.

In a national survey of suspected encephalitis and/or severe illness in children less than 2 years of age with fever and convulsions, 13 (8%) of 156 children were experiencing well-documented primary HHV-6 infection. A unique form of HHV-6-associated encephalopathy has been identified, in which developmentally normal children aged 8 to 15 months experienced a convulsion during the febrile phase of primary HHV-6 infection, followed by several days of disturbed consciousness, and then a brief cluster of clonic convulsions occurs in the day following resolution of the fever and onset of a rash typical of exanthem subitum (Nagasawa et al., 2007). While some of these cases resolved without obvious complication, half were associated with either mental retardation or unilateral hemiplegia. A complex case has been described of a child who developed acute cerebellar dystonia, severe truncal ataxia, and myoclonic dystonia in whom HHV-6A was detected only in the CSF and HHV-6B only in peripheral blood (Borghi et al., 2005). In an interesting report, of 22 herpes simplex encephalitis cases, HHV-6B was simultaneously detected in 3. Two of the 3 dually infected patients died, compared to only 1 of 19 patients with only herpes simplex virus (Tang et al., 1997).

Temporal Medial Lobe Epilepsy

HHV-6B was detected in 11 (69%) of 16 brain resections from patients with mesial temporal lobe epilepsy (Fotheringham et al., 2007b). Viral DNA was detected by PCR, and the virus grew in primary astrocytes cultured from the resected tissues. Viral replication in primary astrocytes led to a decrease in expression of glutamate transporter EAAT-2, which may explain the previously noted defect in glutamate transport in this form of epilepsy.

MS

Several lines of evidence suggest a possible etiologic association between HHV-6 and some cases of MS. These observations have not been universal (Enbom et al., 1997; Martin et al., 1997; Fillet et al., 1998), but the evidence in favor of an association in some populations outweighs the evidence for this being adventitious or due to technical error; it remains to be determined whether the virus is an initiator, an accelerant or pathogenic booster, or a passive passenger. Thus, the virus is present in most brains, can replicate (albeit inefficiently) in primary astrocytes, neurons, and oligodendrocytes, and is associated with neurologic symptoms. More directly, in some studies HHV-6 serologic titers were higher in MS patients than in the control population, including patients with other neurologic diseases, and HHV-6 DNA was detected in CSF or serum from more MS patients than from controls (Sola et al., 1993; Wilborn et al., 1994; Sanders et al., 1996; Soldan et al., 1997; Ablashi et al., 1999). Most intriguingly, HHV-6 antigens were detected in MS plaque regions by immunohistochemistry, with a distribution different than that seen in healthy brains (Challoner et al., 1995). In a 1-year longitudinal study of relapsing-remitting MS, HHV-6 was detected more frequently in PBMCs and serum from patients than from healthy blood donors (81% versus 30%), and the virus was detected more frequently in serum of patients during relapse, but there was no significant association between load and state of disease (Alvarez-Lafuente et al., 2006). Active infection, defined as detection in serum of the viral genome plus lytic transcripts, was more frequently detected during exacerbations. Only HHV-6A was detected in patients meeting this definition. A well-documented case of primary HHV-6 infection, including PCR-positive CSF, was seen in a 13-year-old female who experienced Balo's concentric sclerosis, a demyelinating disease related to MS (Pohl et al., 2005). About 20% of MS patients have intrathecal IgG responses to HHV-6, which was associated with responses to other unrelated viruses (Derfuss et al., 2005). HHV-6 IgM titers against virion proteins did not correlate with MS disease state (Friedman et al., 2005). Levels of the HHV-6 receptor, CD46, can be elevated in MS patients. In an immunoaffinity system, HHV-6A copurified with CD46 from the CSF of MS patients (4 of 42 patients) (Fogdell-Hahn et al., 2005). That HHV-6A and not HHV-6B was detected in this way may reflect this variant having a higher affinity for CD46 than does HHV-6B (Pedersen and Hollsberg, 2006).

Defining the association between HHV-6 and MS remains an interesting and important problem. Ultimate proof of a specific role for HHV-6 in MS will require therapeutic trials that are coupled with refined diagnostic methods that would allow proof of the intended effect. For MS, this is particularly difficult because of the relative inaccessibility of the affected tissue. At present, the clinical utility of HHV-6 diagnostic information remains unclear. As mentioned, high levels of viral activity in the brain do not necessarily result in high levels of viral DNA in the CSF.

Other Disease Associations

Several other connections between HHV-6 and specific diseases warrant brief mention. HHV-6B was identified as the likely cause of two cases of acute liver failure (Cacheux et al., 2005) and fatal adult respiratory distress syndrome (Merk et al., 2005) in immune-competent adults and of seven cases of transient acquired myelodysplasia in immune-competent children (Kagialis-Girard et al., 2006). HHV-6 involvement has been reported in ocular diseases, including acute bilateral uveitis and central retinal vein occlusion (Rhoads et al., 2007; Takizawa et al., 2006).

Many malignancies have been examined for HHV-6 associations, but no conclusive linkages have been identified (reviewed in Braun et al., 1997). The most plausible associations are with some cases of oral cancer (at least in Malaysia), the nodular sclerosis type of Hodgkin's disease, and S100-positive chronic lymphoproliferative disease.

Some studies have described elevated levels of HHV-6 activity or antibodies in patients with chronic fatigue syndrome, but the evidence for an etiologic association is not convincing.

Diagnosis

Classic roseola is diagnosed on a clinical basis, without laboratory confirmation. It may prove important to ascertain

TABLE 4 Analytical methods that distinguish HHV-6A from HHV-6B

Monoclonal antibody reactivity	Restriction endonuclease profiles	Nucleotide sequence analysis	PCR product analysis	Reference(s)
+				Campadelli-Fiume et al., 1993; Chandran et al., 1992; Foa-Tomasi et al., 1995
+	+			Ablashi et al., 1991; Schirmer et al., 1991
+		+		Aubin et al., 1993; Pellett et al., 1993
+	+		+	Dewhurst et al., 1992
		+		Gompels et al., 1993
		+	+	Chou and Marousek, 1994; Yamamoto et al., 1994
			+	Aubin et al., 1994; Boutolleau et al., 2006b; Cone et al., 1996; Drobyski et al., 1993; Gautheret et al., 1996; Hymas et al., 2005; Kidd et al., 1998; Sassenscheidt et al., 2006; Wilborn et al., 1998

+, method used in indicated publication(s).

whether coincident primary HHV-6 infection is the likely cause of illness following routine childhood immunization, avoiding misdiagnosis of an adverse response to the vaccine. Other situations where HHV-6 diagnostics have value include monitoring organ transplant recipients, diagnosis of encephalitis in stem cell transplant recipients, as an adjunct for diagnosis of DIHS, and as part of diagnosing possible cases of measles, mumps, or rubella (Black et al., 1996a; Tait et al., 1996; Oliveira et al., 2003; Davidkin et al., 2005). Because HHV-6 is but one of several agents that may cause a given disease (e.g., encephalitis), multiplex approaches to diagnosis may be required to achieve necessary cost efficiencies. It is not easy to discriminate primary from reactivated or active (lytic) from latent infections, but this can be accomplished by detection of viral transcripts or antigens, or changes in viral load (low during latency) or serologic titers over time. In instances of very high viral load, care must be taken to rule out chromosomally integrated viral genomes. Important issues in HHV-6 diagnosis were recently reviewed (Ward, 2005).

HHV-6 activity can be monitored by culture, PCR, or serology. Specific organ involvement may be best addressed by immunohistochemistry. Commercially available reagents and assays include monoclonal antibodies that react with either or both variants, serologic reagents and assays, and PCR primers. Because of the lack of standardized methods or definitions, the choices of controls or references in such studies are very important. Analysis of single specimens is unlikely to provide useful information. Multiple specimens (e.g., blood, CSF, and hair follicles) from a single time point can be informative, with a more certain diagnosis being made from temporally linked specimens (e.g., acute and convalescent phases).

Virus Detection

HHV-6 viremia seldom occurs other than during periods of frank viral activity, such as during the febrile phase of roseola and during reactivation events in organ transplant recipients. Culture can be accomplished by purification of patient lymphocytes, followed by stimulation with phytohemagglutinin and growth in the presence of IL-2 (Black et al., 1989). Some laboratories culture patient lymphocytes alone, while others cocultivate the activated lymphocytes with similarly purified and activated human umbilical cord blood lymphocytes. Because the cytopathic effect (CPE) in HHV-6-infected cells is indistinguishable from that induced by other viruses, including HHV-7 and HIV, culture isolation must be confirmed, which can be done using monoclonal antibodies, some of which allow variant discrimination (Table 4).

PCR is often done on purified PBMC, but because the virus can inhabit PMNC, it has been proposed that whole blood be used for maximum sensitivity (Palleau et al., 2006). Questions remain about the relationship between the presence of HHV-6 DNA in plasma vs. PBMC vs. whole blood (Achour et al., 2006; Achour et al., 2007). Viral DNA in plasma can be the product of infected cell lysis (the viral DNA is not protected by the nucleocapsid from DNAse treatment), and the amount of DNA in plasma is a fraction of that present in blood cells (Achour et al., 2007). Thus, in a comparison of paired plasma and whole-blood specimens, viral DNA was detected more frequently in the whole blood (Wada et al., 2007). Under these circumstances, plasma PCR can be considered a "detuned" assay, such that if a positive reaction is seen, it is equivalent to a high viral DNA load measured in cells, and might be interpreted as indicating an active infection in which virus is being released. This can allow distinguishing active versus latent infections of lymphocytes that are likely to be of little clinical relevance (Secchiero et al., 1995a; Secchiero et al., 1995b; Chiu et al., 1998; Suga et al., 1998). However, as pointed out by Ogata et al. (Achour et al., 2006), leukocyte counts can be very low in immune-compromised patients in the same periods when HHV-6 activity may be most important, and plasma may be more useful than whole blood for detecting viral activity in the brain, for example, during HHV-6 encephalitis in stem cell transplant recipients. A comparative clinical study is needed to assess the relative value and determine the most appropriate applications of these approaches. In addition to blood, the presence of CMV and HHV-6 DNA in urine predicted severe disease in renal transplant recipients (Ratnamohan et al., 1998). PCR can also be done from saliva collected on absorbent strips (Zerr et al., 2000).

Numerous PCR primer and probe sets have been described, including nested (Cone et al., 1996), nonnested (Aubin et al., 1994; Yamamoto et al., 1994), nonisotopic (Osiowy et al., 1998), quantitative (Secchiero et al., 1995b; Clark et al., 1996; Collot et al., 2002; Gautheret-Dejean et al., 2002; Sashihara et al., 2002; Hymas et al., 2005; Sassenscheidt et al., 2006), and multiplexed (Kidd et al., 1998; Minjolle et al., 1999; Wada et al., 2007). Several primer sets also provide HHV-6 variant discrimination (Table 4). The use of stored quantitative curves was found to be reliable

and cost-effective, and lyophilized standards were more stable than standards stored frozen (Hymas et al., 2005). Clinically validated cutoffs are needed. A less expensive alternative to quantitative PCR methods that are based on amplimer detection by fluorescence resonance energy transfer, is the use of SYBR green (Fernandez et al., 2006), although such assays do not allow for easy discrimination of the HHV-6 variants. Loop-mediated isothermal amplification is an alternative to PCR for rapid and sensitive detection of DNA that has been adapted for detection of HHV-6 (Ihira et al., 2007). Severely immune-compromised patients may not have sufficient lymphocytes to allow for robust quantitation (Achour et al., 2006). Evaluation of viral DNA loads in hair follicles (Ward et al., 2006) or cells from buccal swabs may prove valuable in noninvasively assessing the possibility of ciHHV-6 in tissues that are independent of the illness being studied.

Reverse transcriptase PCR can be used to detect transcripts of viral genes and allow discrimination of active (positive for genome and lytic transcripts) from latent (positive for genome and negative for lytic transcripts) infection. Some of these assays target transcripts of spliced genes, thus ensuring that the amplimer is indeed derived from a viral transcript rather than from stray viral genomes (Norton et al., 1999; Sashihara et al., 2002; Andre-Garnier et al., 2003; Yoshikawa et al., 2003; Pradeau et al., 2006).

An alternative to PCR is an antigenemia assay that detects infected PBMC. With appropriate antibodies, the test can discriminate HHV-6A and HHV-6B infections (Lautenschlager et al., 2000; Volin et al., 2004). Antibodies are available only for lytic cycle proteins, so antigenemia assays are not useful for detecting latent infections. In a comparative study, quantitative PCR and the antigenemia test were both useful for predicting HHV-6 reactivation in stem cell transplant recipients (Wang et al., 2006b).

Antibody Tests

IgM seroconversion occurs 5 to 7 days after onset of symptoms associated with primary infection, peaks at 2 to 3 weeks, and wanes by 2 months. The IgG response becomes detectable within 7 to 10 days and is sustained in most cases for life (Fig. 2). A host of HHV-6 antibody assays has been developed in every standard format, including immunofluorescent (IFA), microtiter-dish-based enzyme immunoassays (EIA), and immunoblots (Black et al., 1989; Black et al., 1996b; Robert et al., 1990; Couillard et al., 1992; Coyle et al., 1992; Chokephaibulkit et al., 1997). Several assays are available commercially. The major applications of HHV-6 serology are for (i) discriminating primary HHV-6 infections from adverse vaccine reactions in young children and (ii) detecting temporal changes in serostatus as part of diagnosing possible HHV-6 involvement in an acute clinical event.

For IgM, a commercially available assay uses a purified viral protein, p38/41, as the antigen (Patnaik et al., 1995). Because healthy individuals can be sporadically positive for HHV-6 IgM, IgM results are more definitive when analyzed in temporally spaced sets.

Given the high prevalence of HHV-6 infections, a single positive IgG result simply places a patient in the vast group of HHV-6 seropositives. No diagnostically useful titer has been identified. Reliable serologic diagnosis of primary infection and of reactivation events requires paired acute- and convalescent-phase specimens; fourfold antibody titer increases are often interpreted as evidence for viral activity. Although appropriately paired specimens may provide a conclusive result, it is neither fast nor convenient; the relevant clinical event may have passed by the time antibody levels rise. Primary infections can be discriminated from reactivation by antibody avidity, which increases with time after infection, and can be assessed by inclusion of urea in the wash solution used following the primary antibody incubation (Ward et al., 1993a; Ward et al., 1993b). Antibody avidity testing may be of particular value in assessing possible HHV-6 infections in recently vaccinated young children.

No serologic assay is variant specific. In addition to the extensive antigenic cross-reactivity between the HHV-6 variants, the variants have sufficient cross-reactivity with HHV-7 to lead to occasional false-positive results unless antigen adsorptions are done or an immunoblot assay is used that is based on non-cross-reactive antigens (Black et al., 1996b). Cross-reaction with HCMV also has been seen in some assays.

Diagnosis of CNS Disease

The diagnosis of HHV-6 encephalitis is strengthened by having multiple lines of evidence that include detection of virus in the CNS compartment. As mentioned, although high levels of viral activity in the brain do not necessarily result in high levels of viral DNA in the CSF, virus loads are elevated in CSF before serum during encephalitis following stem cell transplantation (Fotheringham et al., 2007a). Important considerations include detecting changes in HHV-6 levels (as part of excluding the possibility that the positivity is simply due to chromosomally integrated virus), sampling CSF and not just PBMC or plasma, detecting significant changes in HHV-6 antibody titers following onset of disease, and exclusion of other agents. A change from low to high antibody avidity in the weeks following an event supports the possibility of a primary infection (most likely in young children). If biopsy tissue is available, direct detection of virus in affected tissues can be attempted by PCR or immunohistochemistry.

Therapy

In vitro, HHV-6 isolates growing in T cells are sensitive to ganciclovir, foscarnet, and cidofovir, but not to acyclovir (reviewed in De Bolle et al., 2005). In cultured astrocytes, the virus retained sensitivity to foscarnet but not ganciclovir, which may be relevant to some CNS infections (Akhyani et al., 2006).

A number of case reports and even small studies have described possible positive effects of antiviral intervention for HHV-6-associated CNS disease (Rapaport et al., 2002; Tokimasa et al., 2002; Capouya et al., 2006; Fujimaki et al., 2006; Pohlmann et al., 2007; Vu et al., 2007). HHV-6 loads in the CSF of stem cell transplant recipients with CNS disease decreased during the course of treatment with ganciclovir or foscarnet, suggesting therapeutic benefit (Zerr et al., 2002). HHV-6 levels in saliva decreased in patients treated with ganciclovir (Ljungman et al., 2007) but not in individuals treated with valacyclovir (Hollsberg et al., 2005). Pediatric stem cell transplant recipients who received ganciclovir prophylaxis were less likely to experience HHV-6 reactivation (Tokimasa et al., 2002). Use of pharmacologic monitoring of drug levels might improve the efficacy of the antiviral regimen (Ljungman et al., 2007). The International Herpesvirus Management Forum recommends the use of foscarnet and/or ganciclovir for treatment of HHV-6-associated neurologic disease (Dewhurst, 2004).

In stem cell transplant recipients, a rapid increase in HHV-6 levels in blood associated with engraftment of cells harboring ciHHV-6 can be mistaken for acute infection.

While some suggest that antiviral therapy is inappropriate in such circumstances, others argue that the integrated virus may be pathogenically active in a manner that warrants antiviral use (Boutolleau et al., 2006a; Clark et al., 2006a; Clark et al., 2006b).

Acyclovir and valacyclovir have been evaluated in MS therapy trials, with limited benefits being seen in patients with higher exacerbation rates or longer duration of disease. As might be expected based on its lack of a viral thymidine kinase and low susceptibility in vitro, no effect of valacyclovir therapy was seen on HHV-6 levels in saliva or blood (Hollsberg et al., 2005).

While drug resistance has not been detected frequently, resistance may develop, possibly during therapy intended to target CMV. Ganciclovir-resistant mutants of HHV-6 have been isolated from humans undergoing ganciclovir therapy and following in vitro selection (Manichanh et al., 2001; De Bolle et al., 2002; Safronetz et al., 2003b). Ganciclovir resistance has been mapped to the HHV-6 U69 gene, a homolog of the HCMV UL97 phosphotransferase that activates ganciclovir. It remains to be seen whether the HHV-6 DNA polymerase might also be a site of resistance. A PCR-based system has been developed for rapid assessment of HHV-6 antiviral susceptibility (Isegawa et al., 2007).

HHV-7

HHV-7 was discovered by Frenkel and colleagues in circulating PBMC obtained from a healthy adult (Frenkel et al., 1990). It was subsequently identified as a commensal inhabitant of saliva, with 75% of saliva specimens from adults carrying cell-free infectious virus (Wyatt and Frenkel, 1992; Black et al., 1993; Hidaka et al., 1993). HHV-7 is closely related to the HHV-6 variants and shares many of their molecular and biological properties (reviewed in Yamanishi et al., 2007). HHV-7 is highly and widely prevalent, with infection normally being acquired early in life (Tables 1 and 2). Two disease states associated with the virus are of particular importance: a subset of roseola cases and CMV disease in organ transplant recipients.

Biology

As described above for HHV-6, HHV-7 virion structure is similar to that of other herpesviruses (Fig. 1B) (reviewed in Yamanishi et al., 2007). HHV-7 genomes are more compact than HHV-6 genomes, with a length of approximately 145 kb. Like the HHV-6 variants, HHV-7 genomes are bounded by sequences that resemble mammalian telomeres. Nearly every HHV-7 gene has a counterpart in HHV-6. A significant difference is that both HHV-6 variants encode a homolog of the parvovirus *rep* or *ns*1 gene, but HHV-7 does not. Amino acid sequence identity between HHV-7 and the HHV-6 variants ranges from 22 to 75%, with most genes having identities of 40 to 60%.

The HHV-7 growth cycle has many similarities to those of the HHV-6 variants. It grows best in activated primary cord blood lymphocytes and has been adapted for growth in the SupT1 T-cell line (Berneman et al., 1992; Black et al., 1997; Cermelli et al., 1997; Ablashi et al., 1998). The CPE it induces is indistinguishable from HHV-6 CPE, and it also induces host cell protein synthesis (Black et al., 1997). Cell death in HHV-7-infected cultures can be due to either virus-induced apoptosis or lysis (Secchiero et al., 1997). Unlike HHV-6, HHV-7 uses CD4 as its cellular receptor, as does HIV (Lusso et al., 1994; Yasukawa et al., 1995). Under appropriate conditions in vitro, HIV and HHV-7 can inhibit each other's growth by receptor competition (Lusso et al., 1994). Cocultivation of HHV-7 with fresh peripheral blood lymphocytes that harbor latent HHV-6B can result in reactivation of HHV-6B to a lytic state (Katsafanas et al., 1996). Consistent with this, DNA levels for both viruses were elevated in HIV patients when HHV-6 and HHV-7 were simultaneously present in tissues (Emery et al., 1999). The mechanism of this activity is not known.

Epidemiology

The prevalence of HHV-7 in adults ranges from approximately 60 to 90%. The prevalence of HHV-7 reaches 50 to 70% by the age of 2, with primary infection generally following primary HHV-6 infection (Hall et al., 2006). A subset of HHV-6 and HHV-7 antigens are cross-reactive (Yasukawa et al., 1993; Foa-Tomasi et al., 1994; Black et al., 1996b), which raises the question of whether prior infection with one virus affects infection by the other, even though neutralizing antibodies do not cross-react (Yoshida et al., 2002). Infectious HHV-7 is present in the saliva of most seropositives, thus saliva is likely the primary route of transmission. Because HHV-7 seems to be much more readily available for transmission, it is a mystery why HHV-6B is normally acquired earlier. In addition to saliva, HHV-7 has been detected in about 10% of breast milk specimens and 3 to 10% of cervical and/or vaginal fluids (Fujisaki et al., 1998; Caserta et al., 2007); thus, additional paths of transmission are possible.

Pathogenicity and Clinical Aspects

It is likely that the full spectrum of HHV-7-associated disease has not been defined. To date, primary infection with HHV-7 has been associated with a subset (<10%) of roseola cases and disease similar to that commonly associated with HCMV infections in organ transplant recipients.

Primary Infection

Virologic aspects of primary HHV-7 infection in young children who experience roseola symptoms are similar to those seen for HHV-6B: viremia, clearance, establishment of latent infections in peripheral blood lymphocytes, and development of IgG antibodies (Tanaka et al., 1994; Ueda et al., 1994; Torigoe et al., 1995). Neurologic complications, such as febrile convulsions, have been observed during a third to half of the primary infections that have been described (Torigoe et al., 1995; Torigoe et al., 1996; Clark et al., 1997; Caserta et al., 1998; van den Berg et al., 1999; Hall et al., 2006). It is not clear whether this is a general property of HHV-7 primary infections or is due to the cases reported representing the more severe and less common end of the spectrum. Nonspecific febrile illness, upper respiratory tract illness, and gastroenteritis have each been observed in about 10% of primary HHV-7 infections (Hall et al., 2006).

In a national survey of suspected encephalitis and/or severe illness with fever and convulsions in children less than 2 years of age, primary HHV-7 infection (as defined by changes in antibody avidity in acute- versus convalescent-phase serum specimens) was detected in 15 (10%) of 156 children (Ward et al., 2005); as with HHV-6, these infections occur at ages when they might be clinically misdiagnosed as adverse vaccine reactions. Like HHV-6, primary HHV-7 can be misdiagnosed as measles (Oliveira et al., 2003). After primary infection, HHV-7 DNA continues to be detectable in PBMC for years (Hall et al., 2006). HHV-7 is sometimes reactivated during DIHS (Oskay et al., 2006; Shiohara et al., 2006).

Disease in Immunocompromised Patients

HHV-7 activity and association with disease have been studied in solid-organ, bone marrow, and allogeneic peripheral blood stem cell transplant recipients. A general observation has been that HHV-7 activity peaks near the beginning of the second month after transplant, which precedes the usual onset of HCMV activity by 2 to 4 weeks. Although increased HHV-7 activity has been seen in some studies of transplant recipients, in Japanese and European studies of bone marrow and allogeneic peripheral blood stem cell transplant recipients, HHV-7 activity was seldom associated with disease (Maeda et al., 1999; Volin et al., 2004; Sassenscheidt et al., 2006). Among 60 pediatric stem cell recipients, most episodes of HHV-7 activity were asymptomatic or mild, but reactivation was associated with delayed myeloid engraftment and longer hospitalization (Savolainen et al., 2005). In another study of 59 pediatric stem cell recipients, HHV-7 detection was associated with autologous versus allogeneic transplants and transplantation of peripheral versus umbilical cord blood cells (Chan et al., 2004). Exposure to ganciclovir but not to acyclovir was negatively associated with detecting the virus. The only clinical event associated with HHV-7 was significant: a fatal case of HHV-7-associated brain stem encephalitis.

For solid-organ transplants, no HHV-7 was detected in serum from 33 pediatric liver transplant recipients (Feldstein et al., 2003), but the virus was associated with CMV disease in adults who were CMV-negative at the time of their transplant (Mendez et al., 2001). Although HHV-7 antigenemia was detected in half of 22 lung and heart-lung recipients, its presence was not associated with clinical events (Lehto et al., 2007). In renal transplant recipients, HHV-7 has been associated with rejection and with CMV disease (Osman et al., 1996; Kidd et al., 2000). The risk for CMV disease is highest when both HHV-7 and HCMV were concurrently active.

An observed association between HHV-7 and the recurrent rash illness pityriasis rosea was not confirmed (reviewed in Kempf and Burg, 2000).

Diagnosis

The three circumstances where monitoring HHV-7 activity might have clinical value are in children experiencing febrile rash or neurologic illness following routine childhood vaccination, neurologic illness, and organ transplant recipients. Quantitative PCR or PCR from serum are likely to provide the most useful evidence for viral activity, but their use for this purpose is an area of research, and specific guidelines for their application are not available. Because of the labor and other costs associated with screening for multiple agents, multiplexed PCR assays that allow screening for several agents simultaneously are likely to come into wider use for monitoring organ transplant recipients (Kidd et al., 1998; Minjolle et al., 1999). The high prevalence of HHV-7 makes it difficult to interpret single positive serologic results. Paired acute- and convalescent-phase specimens can help clarify temporal associations between viral activity and disease. Important issues in HHV-7 diagnosis were recently reviewed (Ward, 2005).

Virus Detection

Methods available for detecting HHV-7 include culture, monoclonal antibodies, and PCR. Culture methods for HHV-7 are identical to those used for HHV-6: culture of activated lymphocytes in the presence of IL-2 or coculture of such lymphocytes with additional purified and activated lymphocytes, preferably from human umbilical cord blood (Secchiero et al., 1994; Black et al., 1997). Monoclonal antibodies useful for culture confirmation are commercially available, and an antigenemia assay employing commercially available monoclonal antibodies against HHV-7 gp110/160 has been used to monitor stem cell transplant recipients (Volin et al., 2004). Several PCR systems for HHV-7 have been described, including quantitative and multiplexed methods (Berneman et al., 1992; Secchiero et al., 1995b; Wilborn et al., 1995; Kidd et al., 1996; Kidd et al., 1998; Minjolle et al., 1999; Safronetz et al., 2003a; Sassenscheidt et al., 2006).

Antibody Tests

IFA, EIA, and immunoblot assays have been described for detecting HHV-7 antibodies (Wyatt et al., 1991; Clark et al., 1993; Torigoe et al., 1995; Black et al., 1996b; Huang et al., 1997). False-positive results can be obtained in the IFAs and EIAs due to cross-reactivity with HHV-6. Immunoblot assays using antigens specific for each of the viruses do not cross-react (Black et al., 1996b; Foa-Tomasi et al., 1996), but reagents for such assays are not readily available. The only commercially available HHV-7 serodiagnostic reagents are slides for IFA.

Therapy

In vitro, HHV-7 is susceptible to cidofovir and phosphonoformic acid, less sensitive to ganciclovir than HHV-6 or HCMV, and insensitive to acyclovir and its relatives (Black et al., 1997; Takahashi et al., 1997; Yoshida et al., 1998). Antiviral therapy might be of value for treatment of HHV-7-associated CMV disease in organ transplant recipients (Mendez et al., 2001), but there have been no clinical trials.

HHV-8

HHV-8 was discovered as the etiologic agent of KS following a series of observations and experiments spanning several decades. The geographical distribution of KS in East and Central Africa in the 1960s suggested the involvement of an infectious agent, and in 1972, Giraldo and colleagues detected herpesvirus-like particles in KS biopsy material (Giraldo et al., 1972). In the 1980s there was a dramatic surge in the incidence of KS in conjunction with the emerging AIDS epidemic; the epidemiology of AIDS-associated KS again indicated the involvement of an infectious agent (Beral et al., 1990). In 1994, Chang and colleagues applied representational difference analysis to KS lesion material and identified a novel herpesvirus they named KS-associated herpesvirus, now also known as HHV-8 (Chang et al., 1994). HHV-8 joins EBV, some human papillomaviruses, and hepatitis B virus in the small group of DNA viruses that can cause cancer in humans.

Biology

HHV-8 virions have characteristic herpesvirus morphology (Fig. 3). Based on sequence and biological properties, HHV-8 is classified in the *Rhadinovirus* genus of the gammaherpesvirus subfamily (Moore et al., 1996). Its closest human relative is EBV. The HHV-8 genome is approximately 160 kb long and contains over 85 genes (Russo et al., 1996). In addition to containing the conserved herpesvirus core genes and genes specific to other gammaherpesviruses or rhadinoviruses, HHV-8 has several genes that are of obvious host derivation. These genes encode proteins involved in cell cycle regulation, signal transduction, and modulation of host immune responses (reviewed in Neipel and Fleckenstein, 1999; Moore

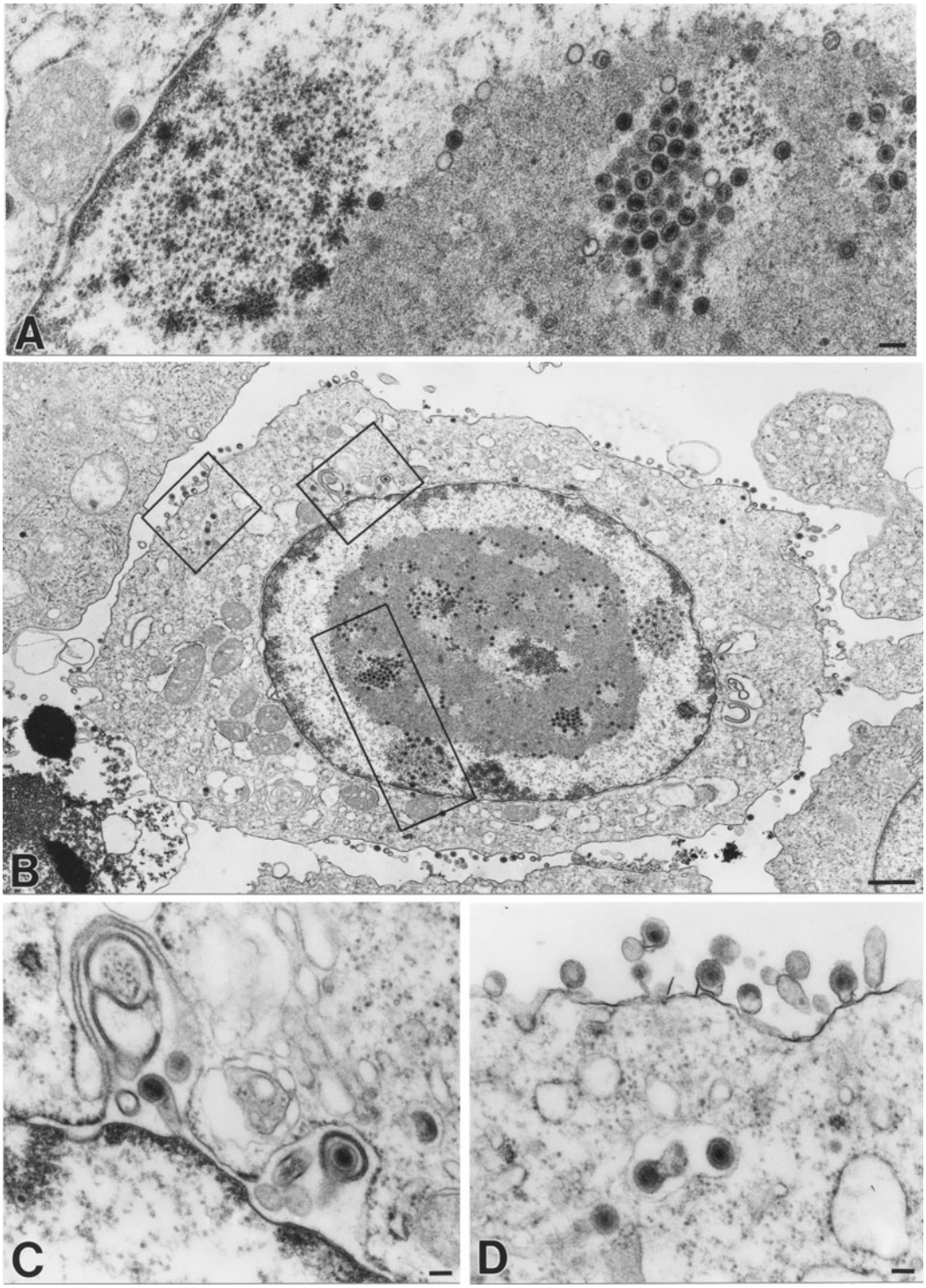

FIGURE 3 HHV-8 virions in BCBL-1 cells incubated with 0.3 mM sodium butyrate for 5 days. Boxed areas in panel B are shown at higher magnification in panels A, C, and D. (B) Induced cells show signs of lytic infection including condensed and marginated chromatin and numerous nuclear inclusions that contain maturing virus particles (A). Virions in membrane-bound vesicles associated with the nuclear (C) and cytoplasmic (D) membranes are shown. Culture material was provided by Margaret Offermann (Emory University) and Jodi Black (Centers for Disease Control and Prevention [CDC]) (Yu et al., 1999). Electron microscopy and photography were by Cynthia Goldsmith, CDC. Bars = 1 μm (B) and 100 nm (A, C, and D).

and Chang, 2003). In vivo, HHV-8 is most often in a latent form in which very few viral genes are expressed. The K1 gene, which maps at the extreme left end of the HHV-8 genome, is a transforming gene whose product is a membrane-associated glycoprotein that functions in cell signaling (Lee et al., 1998). The nucleotide and amino acid sequences of K1 form five major genotypes that diverge by as much as 30% among viral strains collected around the world and reflect global patterns of human migration (Cook et al., 1999; Hayward, 1999; Meng et al., 1999; Zong et al., 2002). Hayward and colleagues have identified two major and highly divergent alleles of the K15 gene (Hayward, 1999), which maps at the right end of the genome. Although more genetically conserved than the K1 loci, K15 alleles also have been useful for studies on genetic variability and evolution of HHV-8.

No successful animal model for the study of HHV-8-associated disease has been reported. HHV-8 has a surprisingly broad host range for virus entry and establishment of latency, but efficient replication and serial passage of clinical isolates in cell lines has not been demonstrated (Blackbourn et al., 2000; Renne et al., 1998). Some success at overcoming these barriers has been achieved via ectopic expression of the HHV-8 replication and transcription activator protein encoded by open reading frame (ORF) 50 (Bechtel et al., 2003).

Human B-cell lines naturally infected with HHV-8 have been generated from PEL. Such cell lines that harbor HHV-8 but not EBV are in wide use for studying HHV-8 molecular and cellular biology and for use as serodiagnostic reagents (Cesarman and Knowles, 1999; Bechtel et al., 2005). Under normal growth conditions for PEL cell lines, the virus is latent in most cells. In response to treatment with stimulants such as phorbol ester (tetradecanoyl phorbol acetate) or sodium butyrate, lytic replication is induced in 10 to 30% of cells.

Four HHV-8 proteins are consistently detected in latently infected cells: kaposin (ORF K12), v-FLIP (ORF 71), v-Cyclin (ORF 72), and the latency-associated nuclear antigen (LANA; ORF 73), with kaposin expressed at higher levels than the other latent proteins (Jenner et al., 2001; Pearce et al., 2005). Of the numerous HHV-8 lytic cycle genes, the most abundantly expressed is the T1.1 or *nut-1* transcript (Zhong et al., 1996). The high copy numbers of the kaposin and T1.1 transcripts during latent and lytic infection, respectively, make them useful targets for in situ hybridization (ISH).

Epidemiology

HHV-8 differs from the other human herpesviruses by having a very low seroprevalence in much of the world; high HHV-8 infection rates are found only where KS is more prevalent. Regions with the highest HHV-8 seroprevalence (40 to 60%) are in sub-Saharan Africa where KS is a very common and aggressive cancer in men, women, and children. Moderate seroprevalence (10 to 30%) is observed in Eastern Mediterranean countries, including Italy, Greece, and Israel, where KS is typically a nonaggressive, indolent disease that most often affects the lower extremities of middle-aged and older men. Low HHV-8 seroprevalence (<5%) is seen in North America and Northern and Western Europe, where the occurrence of KS is almost entirely limited to homosexual and bisexual men with AIDS, plus a small portion of organ transplant recipients (reviewed in Schulz, 1999; Cohen et al., 2005). The uneven distribution of HHV-8 is surprising in view of substantial genetic evidence that HHV-8 was not recently introduced to humans but has long been part of human biology. In countries where KS is endemic, HHV-8 seroprevalence is distributed across the population in the absence of identified behavioral risk factors. In countries where KS is rare, HHV-8 seroprevalence is highest in KS patients, next highest in HIV-positive patients who are men who have sex with men (MSM), then HIV-negative MSM, with few healthy blood donors testing positive (Fig. 4).

Transmission

Transmission of HHV-8 is not entirely understood but appears to be via saliva for both casual and sexual transmission. In African countries where it is endemic, the most important mode of transmission for HHV-8 appears to be horizontal

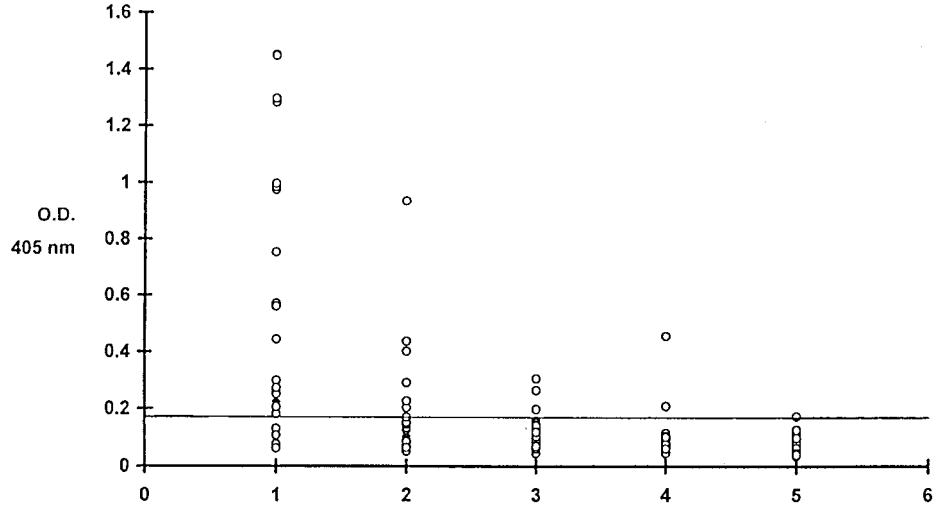

FIGURE 4 Relative HHV-8 antibody reactivity measured by enzyme-linked immunosorbent assay with recombinant ORF 65 protein among various groups: 1, patients with AIDS KS; 2, HIV-infected homosexual men without KS; 3, United Kingdom blood donors; 4, intravenous drug users; 5, hemophiliacs. O.D., optical density. Figure courtesy of Thomas Schulz, Hannover Medical School, and The Lancet Ltd. (Simpson et al., 1996). Reprinted with permission from Elsevier.

and casual, beginning early in childhood from mother to child and among siblings and other household contacts (Bourboulia et al., 1998; Mayama et al., 1998; Gessain et al., 1999). Transmission from mother to child in utero or from breastfeeding is rare and does not contribute substantially to the spread of the virus in populations of areas where it is endemic (Brayfield et al., 2003; Brayfield et al., 2004).

In countries where it is not endemic, HHV-8 is concentrated among MSM with risk factors that are clearly sexual; HHV-8 seroprevalence correlates with the duration of homosexual lifestyle, the number of male partners, and a history of other sexually transmitted infections (Martin et al., 1998; Melbye et al., 1998; Renwick et al., 1998; Blackbourn et al., 1999; Grulich et al., 1999). HHV-8 DNA has been detected in saliva (Blackbourn and Levy, 1997; Koelle et al., 1997; Blackbourn et al., 1998; Pellett et al., 1999) and less frequently in semen (Blackbourn and Levy, 1997). Several reports have identified linkages between HHV-8 transmission and oral-anal contact, but this is not conclusive, nor is there a convincing linkage to any specific sexual practice. HHV-8 DNA also has been detected in cervicovaginal specimens of HHV-8-seropositive women (Calabro et al., 1999; Whitby et al., 1999), although heterosexual HHV-8 transmission appears to be rare in regions where it is not endemic (Engels et al., 2007) for reasons that are not understood.

The most notable new development regarding the transmission of HHV-8 has been in the realm of blood transfusion. In 2001, Cannon and colleagues found that seropositivity was strongly associated with frequency of injection drug use, providing the first clear evidence for blood-borne transmission of HHV-8 (Cannon et al., 2001). Subsequently, other reports confirmed that HHV-8 can be transmitted by injection drug use (Atkinson et al., 2004) and demonstrated HHV-8 infection by blood transfusion (Hladik et al., 2006) in Uganda, although the risk was low relative to other blood-borne pathogens. Virus was transmitted from about 3% of the HHV-8-seropositive blood units. In the United States, HHV-8 seroprevalence among blood donors is <5% (Pellett et al., 2003) and there is no evidence for KS developing in transfusion recipients; therefore, universal screening of the blood supply for HHV-8 is not likely to be implemented.

The risk of developing KS following organ transplantation is low compared to other posttransplant complications but increases with the HHV-8 prevalence in a region. The reported incidence of posttransplant KS ranges from 0.5 to 5% depending on the regional prevalence of HHV-8 and on the type of organ transplanted, with the highest rates being associated with renal transplants. There is strong evidence that transplant-associated KS in higher seroprevalence regions (Mediterranean Europe) is primarily due to virus reactivation in the recipient and not infection from the donor (Frances et al., 2000; Parravicini et al., 1997; Andreoni et al., 2001). However, transmission of HHV-8 from the organ donor to the transplant recipient can occur and lead to the development of KS (Regamey et al., 1998; Barozzi et al., 2003). Reduction of immunosuppressive therapy is effective for treating KS but increases the risk for graft rejection and patient mortality; thus, screening of organ donors and recipients for their HHV-8 status should be considered.

Pathogenicity and Clinical Aspects

Primary Infection

Primary HHV-8 infection is rarely observed, and symptoms, when present, are likely mild and nonspecific. In a study of 5 adults, 3 experienced mild lymphadenopathy contemporaneous with seroconversion, one had a localized rash, and one was asymptomatic (Wang et al., 2001). Symptoms of primary infection may be more apparent in young children, as suggested by a study in Egypt where 5 of 6 children with suspected or confirmed primary HHV-8 infection were febrile and had a maculopapular skin rash (Andreoni et al., 2002). The triggers that lead to development of KS in a subset of HHV-8-positive individuals have not been identified.

KS

KS is a neoplasm that originates in endothelial tissues and is characterized by reddish-brown plaque or nodular lesions (Color Plate 7). Lesions most commonly localize to the skin of the extremities and trunk but can also occur in internal organs. Groups of lesions can expand and coalesce into large tumor masses. Pathologically, KS lesions are characterized by the presence of cells with a spindle-like appearance, extensive networks of vascular slits, extravasated red blood cells, and purplish pigmentation from hemosiderin. Not a classic cancer, KS is a proliferative condition intermediate between the benign and malignant categories.

HHV-8 DNA, transcripts, and proteins are present in the spindle cells of KS lesions, including the earliest stages of lesion development (Boshoff et al., 1995; Li et al., 1996; Staskus et al., 1997; Dupin et al., 1999; Staskus et al., 1999). Most HHV-8-containing cells express latent antigens (Color Plate 7) or transcripts, while lytic replication takes place in a small subset of cells.

Epidemiologic, pathologic, and molecular data make it clear that HHV-8 is the etiologic agent of all forms of KS (reviewed in Schulz, 2006, and summarized in Table 5), but the virus alone is insufficient to cause KS. An important cofactor for the development of AIDS- and transplant-associated KS appears to be severe immunosuppression. Cofactors for the development of classic and African KS are less clear, but immunosuppression secondary to age (classic) and malnourishment (African) is thought to play a role. In many parts of Africa, KS has been recognized for decades as a common tumor that affected men more than women and

TABLE 5 Epidemiological forms of KS

Form	Population	Associated with immune impairment	Time period first described
Classic	Older individuals of Mediterranean or Eastern European Jewish ancestry	Possibly	1872
African endemic	All age groups in equatorial Africa	Possibly	1950s
Transplant related	Organ transplant recipients	Yes	1960s
AIDS related	HIV type 1-infected individuals	Yes	1980s

children. During the AIDS epidemic, the incidence of KS dramatically increased, and it is now the most common malignancy in all segments of the population. The prevalence of KS and the associated morbidity and mortality will remain high in Africa until treatment for HIV infection becomes widely available.

KS is the most common malignancy in AIDS patients in the United States, with 15 to 25% of male AIDS patients in the United States having KS as their AIDS-defining illness and approximately 20% of male patients experiencing KS during the course of their AIDS (Jones et al., 1999). The time from HHV-8 infection to onset of KS is 2 to 10 years for AIDS patients compared to decades for classic KS (Martin et al., 1998). The incidence of KS among AIDS patients in the United States and Western Europe has dropped dramatically since 1996 after widespread adoption of highly active antiretroviral therapy (HAART). The main effect of HAART on KS is indirect due to elevated CD4 counts which result in lower levels of HHV-8 in peripheral blood (Jacobson et al., 2000).

PEL

PEL belongs to a class of non-Hodgkin's B-cell lymphomas known as body cavity-based lymphoma. Such tumors occur as pleural, pericardial, or peritoneal lymphomatous effusions in the absence of a solid tumor mass; almost all cases of PEL harbor HHV-8 (Cesarman et al., 1995; Cesarman and Knowles, 1999). PEL are rare even in AIDS patients, constituting less than 1% of all AIDS-associated lymphomas in the United States (Ablashi et al., 2002). Distinguishing properties of PEL are summarized in Table 6. As mentioned above, much of what we know of the molecular biology of HHV-8 has been learned from cell lines derived from these tumors. Given the several mechanisms by which HHV-8 can transform cells, it is likely that the virus is the direct cause of PEL, although considering how uncommon PEL are, it is clear that other factors are involved in their pathogenesis.

MCD

MCD is a multicentric lymph node angiofollicular hyperplasia characterized by vascular proliferation in germinal centers. It occurs primarily in AIDS patients, sometimes linked with KS or non-Hodgkin's lymphoma; in this context, MCD lesions nearly always contain HHV-8 (Soulier et al., 1995). MCD is much less common in HIV-negative individuals, and HHV-8 is associated with half or fewer of such cases (Katano et al., 2000; Parravicini et al., 2000). HHV-8 is present in the hyperplastic regions of MCD lesions in a subset of patients with MCD. Three rapidly progressing and severe MCD cases were identified over a brief interval in one hospital in patients who recently initiated HAART (Zietz et al., 1999), raising the possibility that some aspect of the antiretroviral therapy promoted the disease. Thus far, there have been no indications that this represents anything other than a sporadic outbreak.

TABLE 6 Common properties of PEL[a]

Lymphomatous effusion without contiguous tumor mass in pleural, peritoneal, and/or pericardial cavity
Seldom disseminated beyond cavity of origin
Cell morphology bridges large cell immunoblastic lymphoma and anaplastic large cell lymphoma
Expression of CD45 and activation-associated antigens
Infrequent expression of B-cell-associated antigens
Clonal immunoglobulin gene rearrangements indicating B-cell origin
Infrequent c-*myc* rearrangements or bcl-2, ras, and p53 alterations
Frequently coinfected with EBV
HHV-8 DNA in every tumor cell

[a]Based on Cesarman and Knowles, 1999.

Other Disease Associations

Numerous diseases have been studied for possible associations with HHV-8. Apart from KS, PEL, and MCD, none has emerged with a clear etiologic link to HHV-8. One possible association that received a lot of attention was between HHV-8 and multiple myeloma. Subsequent PCR, serologic, and epidemiologic studies from many laboratories failed to corroborate the initial observations, and the association now seems unlikely (reviewed and discussed in Berenson and Vescio, 1999; Tarte et al., 1999). More recently, a high-profile report of HHV-8 involvement in primary pulmonary hypertension (Cool et al., 2003) was not supported by subsequent studies (Daibata et al., 2004; Henke-Gendo et al., 2004; Katano et al., 2005; Laney et al., 2005). Likewise, reports of HHV-8 associations with sarcoidosis and angiosarcoma were followed by several reports of no involvement of HHV-8 with these diseases. Other disease associations for HHV-8 have been reported for various lymphomas, cutaneous neoplasms, encephalitis, and Bowen's disease, but investigations have been too few or the diseases too rare to rigorously confirm or refute any involvement of HHV-8. Some of these putative disease associations have been based on nested PCR or a single serology test. Such results need to be interpreted cautiously and confirmed with other methods and by other laboratories.

Diagnosis

The major application of HHV-8 diagnostic tests is in research studies of epidemiology, transmission, and new disease associations. Appropriate and useful clinical applications for HHV-8 testing would include evaluation of questionable lesions to confirm or rule out KS, prediction of KS development, monitoring the effects of KS therapy, and possibly donor-recipient matching in organ transplantation.

A major predictor for KS development is HHV-8 seropositivity in HIV-infected individuals. Viral load testing by quantitative PCR is useful for monitoring the effect of KS therapy. HHV-8-positive organ donors can be identified by serologic testing, which may be of greater value in regions with higher HHV-8 prevalence.

Serology

The first HHV-8 serology tests were IFA based on HHV-8-infected PEL cell lines that, with modifications, are still widely used today. PEL cells treated with a virus-inducing agent such as tetradecanoyl phorbol acetate or sodium butyrate are used for the detection of antibodies against various lytic cycle proteins (Lennette et al., 1996), and cells not treated with an inducing agent are used for the detection of antibodies against the main latent cycle protein, LANA (Kedes et al., 1996; Simpson et al., 1996). The lytic IFA can detect HHV-8 antibody in >95% of persons with KS but, at low serum dilutions, has reacted with 10 to 20% of serum specimens from blood donors, which likely overstates blood donor seroprevalence. Modifying the lytic IFA by using a higher serum dilution (1:40 instead of 1:10) retains its sensitivity and improves its specificity (Smith et al., 1997; Pellett et al., 1999; Dollard et al., 2005). IFA or immunoblots based on

the LANA protein are somewhat less sensitive for identifying KS patients as seropositive and are much less sensitive for detecting infection in healthy individuals (Rabkin et al., 1998; Enbom et al., 2000; Schatz et al., 2001). Among the dozen or so immunogenic viral proteins identified (Chandran et al., 1998; Raab et al., 1998), three have proven useful in serologic assays: ORF K8.1, ORF 65, and ORF 73 (LANA). K8.1 is a lytic cycle gene encoding a glycoprotein located in the viral envelope with no homolog in other herpesviruses (Raab et al., 1998). ORF 65 is a lytic cycle gene that encodes a viral capsid protein that is the homolog to the EBV capsid protein BFRF3 (Nealon et al., 2001). ORF 73 encodes a latent cycle protein (LANA) required for replication and persistence of viral DNA (Ballestas et al., 1999). Several enzyme-linked immunosorbent assay-based tests have been developed for HHV-8 based on the K8.1 (Engels et al., 2000; Spira et al., 2000) and ORF 65 (Simpson et al., 1996; Chatlynne et al., 1998; Pau et al., 1998) viral proteins.

The performance of a wide range of HHV-8 serology tests has been investigated in several multicenter studies in which identical panels of sera from different demographic groups were blindly tested in independent laboratories (Cordero et al., 1997; Enbom et al., 2000; Schatz et al., 2001; Pellett et al., 2003). Some HHV-8 assays show good sensitivity and interassay agreement with sera from KS patients, but interassay agreement is consistently poor with sera from subjects at low risk for HHV-8 infection. However, the low level of test concordance may be less a problem of test performance but rather may be due to the nature of the immune response to HHV-8. Healthy subjects typically have very low antibody titers that are near the threshold of detection, and epitope recognition is often limited to a subset of epitopes that can differ from person to person (Simpson et al., 1996; Chandran et al., 1998; Biggar et al., 2003).

Some HHV-8 testing strategies have performed well in epidemiologic studies that examine relative differences between groups (Hladik et al., 2006; Engels et al., 2007), and some individual tests consistently perform better than others in multicenter studies, but no HHV-8 serologic test has the >98% accuracy seen with many commercial antibody tests for other infectious agents. Evaluation of HHV-8 test accuracy is challenging, since most infections are asymptomatic and it is difficult to identify controls known to be HHV-8 uninfected.

PCR

In healthy individuals, HHV-8 viral loads in blood and saliva are usually very low; therefore, PCR is not the best method to detect infection. HHV-8 PCR has been used primarily to study transmission, correlates of viral load with disease, and viral genotypes. Numerous PCR primer sets have been described that appear to perform well (Table 7), although the first primer pairs described by Chang and colleagues in the initial description of HHV-8 are still among the most widely used (Chang et al., 1994). HHV-8 DNA is present in high concentrations in KS biopsy material, but collection of biopsy material is painful for the patient and is not necessary for routine diagnosis. Viral DNA is present in the saliva and blood of a portion of individuals with KS and less commonly in infected persons without KS, so these are the specimens of choice for many studies.

TABLE 7 Genomic targets for HHV-8 diagnostic PCR

Viral gene	PCR type	Reference
Orf 26	Nested	Chang et al., 1994
Orf 26	Nested	Whitby et al., 1995
Orf 25	Nested	Boshoff et al., 1995
Orf 26	One-step	White and Campbell, 2000
Orf 25	One-step	Stamey et al., 2001
K6 (vMIP)	One-step	de Sanjose et al., 2002

PCR can be performed in a one-step method or as nested PCR in which the PCR products of the first amplification are subject to a second amplification using primers that are situated internal (or nested) to the first set of primers. Nested PCR is sometimes more sensitive, but is more prone to false-positive amplification, and results need to be interpreted with caution. Quantitative PCR methods have revealed strong correlations between HHV-8 viral load and the presence and progression of KS (Whitby et al., 1995; Campbell et al., 2000; Quinlivan et al., 2002; Cannon et al., 2003; Engels et al., 2003; Laney et al., 2007).

ISH and IHC Staining

ISH and immunohistochemical (IHC) staining have been useful for identifying the precise location of virus and viral proteins in infected tissues. ISH and IHC staining have been used to study the expression and location of latent versus lytic transcripts and viral proteins (Staskus et al., 1997; Dupin et al., 1999; Katano et al., 2000; Parravicini et al., 2000). More recently, IHC staining using monoclonal antibodies against the HHV-8 latent protein LANA has proven useful to confirm the diagnosis of KS in lesions with nontypical pathologies and to rule out KS in the case of other spindle cell lesions of unknown origin (Color Plate 7) (Cheuk et al., 2004; Patel et al., 2004; Hammock et al., 2005).

Prevention and Therapy

Although the routes of HHV-8 transmission have not been unambiguously defined, safe-sex practices such as the use of latex condoms during sexual intercourse are recommended to reduce the risk of infection (Centers for Disease Control and Prevention, 1999). Oral fluids are likely vehicles for transmission, but effective strategies have not been identified for reducing their exchange. An effective vaccine against HHV-8 would provide the best protection, but one is not on the horizon.

Dramatic declines in AIDS-associated KS began in 1996 with the introduction of HAART for the treatment of AIDS, which led to a six- to eightfold decrease in the incidence of new KS and regression of existing KS in a majority of patients (Engels et al., 2006; Di Lorenzo et al., 2007). The mechanism for the therapeutic effect of HAART on KS is largely indirect by inhibition of HIV replication and subsequent recovery of the immune response to HHV-8, although the protease inhibitors in HAART may also directly inhibit HHV-8-induced angiogenesis (Cattelan et al., 2001; Sgadari et al., 2002; Stebbing et al., 2003).

Despite the declines in the incidence of KS with HAART therapy, KS remains common among individuals with AIDS. Systemic chemotherapy is warranted in patients with rapidly progressive KS marked by widespread skin involvement, symptomatic visceral involvement, or extensive oral KS. Several chemotherapeutic agents have been reported for the treatment of KS, but liposomal anthracyclines appear to be the best option for most patients with widely disseminated KS and show efficacy and tolerance in combination with HAART (Osoba et al., 2001; Lichterfeld et al., 2005).

Radiotherapy is effective in the management of localized KS lesions that are large or that cause discomfort, such as those on the feet. Most irradiated KS lesions show significant shrinkage of the tumor with acceptable tolerance; however, oral lesions are more likely to show adverse reactions to irradiation (Kirova et al., 1998; Yildiz et al., 2006).

Identification of HHV-8 as the cause of KS offers the possibility of specific therapy directed at the virus. In vitro, HHV-8 lytic replication is highly sensitive to cidofovir and is also sensitive to ganciclovir and foscarnet (Kedes and Ganem, 1997; Medveczky et al., 1997). There is some evidence of possible therapeutic benefit from these drugs (Costagliola et al., 1995; Jones et al., 1995; Jones et al., 2000; Glesby et al., 1996; Mocroft et al., 1996; Luppi et al., 2002; Little et al., 2003), but further trials are needed.

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Poxviruses†

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29

LABORATORY PROCEDURES FOR DETECTING POXVIRUSES

Introduction

Smallpox, a disease caused by *Orthopoxvirus variola*, was one of the most feared illnesses of mankind. In 1798, Edward Jenner described that milkmaids with evidence of prior infection with cowpox (caused by *Orthopoxvirus cowpox*) were immune to infection with smallpox (variola virus). Smallpox vaccine, *Orthopoxvirus vaccinia*, was subsequently used extensively for routine vaccination against variola virus. Through an intensive vaccination campaign, coordinated by the World Health Organization (WHO), naturally occurring variola virus was declared eradicated in 1980. Final destruction of all declared laboratory stocks of variola (consolidated at two WHO reference centers) was postponed by the World Health Assembly while research and development of future vaccines, rapid diagnostics, and antiviral therapies were investigated. These modalities are also of interest in recognition and control of emerging zoonotic orthopoxviruses (*Orthopoxvirus monkeypox*, *Orthopoxvirus cowpox*, and *Orthopoxvirus vaccinia*).

Historically poxvirus infections were laboratory confirmed by a combination of approaches including pock morphology on chicken embryo chorioallantoic membranes (CAMs), serologic reactivity, and electron microscopy (EM). It is critical to be able to identify which orthopoxvirus is causing disease, especially when the clinical presentations may be very similar or indistinguishable but the disease severity, mechanisms of disease acquisition, and transmissibility between humans vary greatly, such as with variola virus and monkeypox virus. Most routine serologic methods cannot differentiate between orthopoxvirus species, nor can EM. Laboratory diagnosis of species historically employed biologic characterization of the pock morphology on CAMs of chicken embryos (Table 1) (Marennikova and Shchelkunov, 2005). Diagnosis of parapoxvirus and yatapoxvirus infections was accomplished by EM of lesions in combination with clinical and epidemiologic findings. Finally, the presence of molluscum bodies in a squash preparation of lesion material or a hematoxylin and eosin-stained section of a biopsy specimen was considered pathognomonic for molluscum contagiosum infection. More recently, additional sensitive, rapid, and cost-effective diagnostic assays have become available.

Collection, Handling, and Storage of Specimens

In the United States, poxvirus diagnostic specimens should be evaluated by public health laboratories, many of which are members of the Laboratory Response Network (LRN). The LRN is a network of about 150 laboratories distributed throughout the United States and internationally that are equipped and trained to perform diagnostic assays for orthopoxvirus infections (for more information on the LRN, go to http://www.emergency.cdc.gov/lrn/factsheet.asp). An algorithm was created to assist clinicians in the identification of patients infected with smallpox and differentiation from other pustular rash illnesses (http://www.emergency.cdc.gov/agent/smallpox/diagnosis/pdf/spox-poster-full.pdf). The algorithm includes rule outs for common rash-like illnesses that may be confused with orthopoxviral infections and provides clinical clues that help differentiate them. In the absence of endemic smallpox, if an infection has a high suspicion of smallpox after careful clinical evaluation, the nearest government health department and the Federal Bureau of Investigation (FBI) must be immediately notified and samples sent simultaneously for variola virus diagnostic testing at an LRN laboratory and the WHO Collaborating Center for Smallpox and other poxvirus infections at the Centers for Disease Control and Prevention (CDC) in Atlanta, GA. Final confirmation and characterization of variola virus from initial smallpox cases would require additional examination (including DNA sequence analysis) at the CDC (for more details on smallpox definition and diagnostics, etc., go to http://www.emergency.cdc.gov/agent/smallpox/).

Appropriate biosafety level precautions must be taken for the handling, transport, and processing of suspect infected lesion material (Nakano, 1979; Ropp et al., 1999). Guidelines have been published for sample collection from patients believed to be infected with an orthopoxvirus (http://www.emergency.cdc.gov/agent/smallpox/vaccination/vaccinia-specimen-collection.asp and http://www.cdc.gov/ncidod/monkeypox/diagspecimens.htm). Skin lesions are typically the specimen of choice for diagnosis for any poxvirus

†This chapter includes significant updates from the previous chapter authored by R. Mark L. Buller.

TABLE 1 Historic standards for diagnosis of poxviruses[a]

Standard	Orthopoxvirus				Yatapoxviruses and parapoxviruses	Molluscum contagiosum virus	Herpesviruses
	Cowpox	Monkeypox	Variola	Vaccinia			
Lesions on chorioallantoic membranes	Majority of pocks are flat, poorly defined, hemorrhagic; isolated white pocks	Small pocks with central hemorrhage; isolated larger white pocks	Monomorphic white, sharply defined dome-like pocks	Large, white or gray flat pocks	Do not form	Do not form	Varicella-zoster does not form lesions; herpes simplex forms small whitish lesions
Hemagglutination activity	Absent or weakly expressed	High	Absent or weakly expressed	Marked	Absent	Absent	Absent
Scarification reaction in rabbits	Papulopustular rash with hemorrhage and edema	Papulopustular rash, sometimes with a generalized process	Absent	Papulopustular rash	Absent	Absent	Absent
Intracellular inclusions	Cytoplasmic A and B type	Cytoplasmic B type	Cytoplasmic B type	Cytoplasmic B type	Cytoplasmic B type	Cytoplasmic, acidophilic granular masses (molluscum bodies)	Nuclear

[a]Adapted from Marennikova and Shchelkunov, 2005.

infection. Virions are usually present in this material and can remain viable even after several weeks of storage with or without refrigeration. Because lesion material may be analyzed by several different methods, it is important that sufficient quantities of specimen are collected for submission. In brief, for suspected orthopoxirus, parapoxvirus, and yatapoxvirus infections, at least two biopsy specimens should be obtained and may be acquired from lesions at the macular-papular, vesicular-pustular, and/or crusting stages. During the vesicular-pustular stage, the fluid (including cells from the base of the lesion where the virus is often found in high concentration) can be collected onto dry swabs, as a thick droplet or touch prep onto glass slides, or on plastic-coated EM grids (Nakano, 1979; Ropp et al., 1999). Carrier media should not be added to any of the specimens, as it may dilute specimens for EM. The specimens can be stored for a short time at 4°C; however, –20°C or –70°C are preferable for longer-term storage. An in-depth guide for the collection and shipping of potential smallpox samples is available online (http://www.emergency.cdc.gov/agent/smallpox/response-plan/files/guide-d.pdf).

International and/or local shipping regulations must be followed when preparing suspect etiologic agents for shipment. Guidelines and links to these regulations can be found at http://www.cdc.gov/ncidod/monkeypox/pdf/specimenguide.pdf. The specimens should be initially packaged in a rigid receptacle matched to the size of the specimen with a leakproof cap. It is critical to individually contain specimens to prevent cross-contamination. The primary receptacles should then be wrapped in enough absorbent material to absorb their entire contents should a leak occur and placed into a watertight, crush-resistant outer container. This secondary package should then be surrounded by dry ice or wet ice within an outer package that meets requirements of the U.S. Department of Transportation and International Air Transport Association (IATA) (http://www.iata.org/whatwedo/cargo/dangerous_goods/index.htm) regulations.

Laboratory Diagnostic Methods

Identification and differentiation of poxvirus species have been carried out using a variety of analytical approaches, including virus growth, histology, EM, and serology; however, analysis of lesion material for poxvirus DNA by PCR is currently the most rapid and sensitive primary laboratory diagnostic technique.

Growth of Virus

Traditionally, the CAM of the 12-day-old chicken embryo has been used to culture the orthopoxviruses variola, monkeypox, vaccinia, and cowpox, since all of these viruses grow well on this substrate and can be tentatively differentiated from one another by their characteristic pock (lesion) morphology (Table 1); however, this approach does not support the growth of parapoxviruses, yatapoxviruses, and molluscum contagiosum virus (Marennikova and Shchelkunov, 2005). In vitro cell culture is another approach used to cultivate poxviruses. Orthopoxviruses produce a cytopathic effect on a variety of cell cultures, including immortalized African green monkey kidney cells (Fig. 1). Parapoxviruses orf and pseudocowpox virus have been propagated in cells of ovine and bovine origin, respectively. Although *Yatapoxvirus tanapox* can be grown in rhesus monkey kidney cells ($LLC\text{-}MK_2$), the process is rather inefficient, as only 20 isolations were made from 145 specimens in which Tanapox virus virions were detected by EM (Nakano, 1979). Tanapox virus has been more efficiently propagated in owl monkey

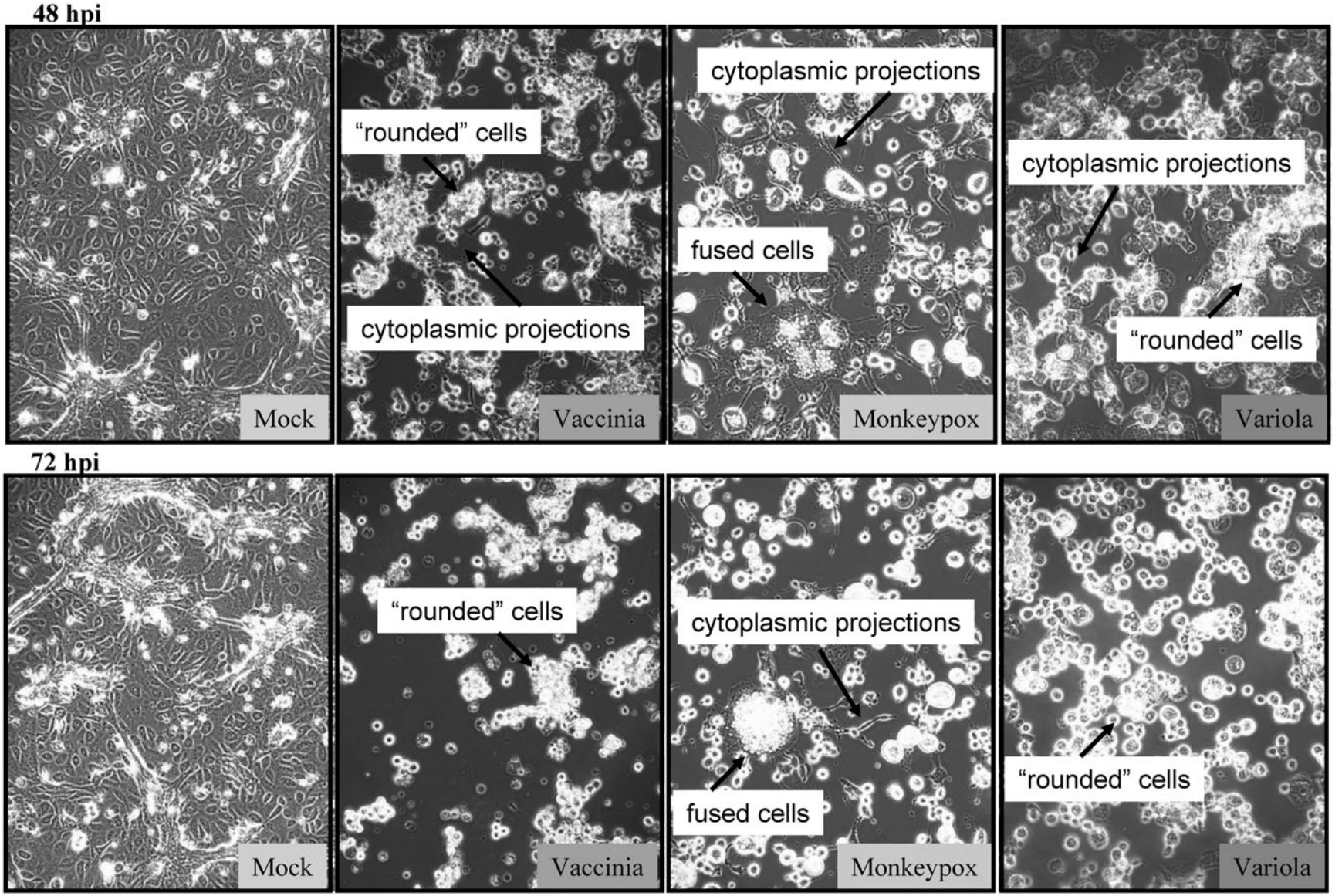

FIGURE 1 Cytopathic effect of orthopoxvirus infection within tissue cell culture. African green monkey kidney cells (BSC-40) were infected at a low multiplicity (multiplicity of infection = 0.01) to mimic what might be found within a clinical specimen. Cells were either mock infected or infected with one of the following orthopoxviruses: vaccinia, monkeypox, or variola. Cells were observed daily, and photographs were taken. The number of hours postinfection (hpi) and characteristics of cytopathic effect are denoted. Magnification, ×10.

kidney cells (Mediratta and Essani, 1999). Molluscum contagiosum virus has yet to be propagated using either CAM or cell culture. Because virus cell culture growth assays are laborious, expensive, and slow, can be relatively insensitive, and do not differentiate among individual species of poxvirus within a genus, PCR analysis of virion DNA from lesion material has largely supplanted these assays for preliminary laboratory poxvirus genus and species identification. However, viral culture analysis remains the sole method for determination of whether the sample contains viable virus. In the absence of endemic smallpox, viral culture should never be attempted if a sample is suspected to contain variola virus unless at one of the two WHO collaborating centers sanctioned for live variola virus manipulation.

Histology

Histological examination of poxvirus lesions can be especially informative with both parapoxvirus and molluscum contagiosum virus infections. Parapoxvirus lesions exhibit an epidermis which is hyperplastic with strands of epidermal keratinocytes penetrating into the dermis. The molluscum contagiosum lesion has pear-shaped lobules filled with intracytoplasmic Henderson-Paterson or molluscum bodies. In practice, histology is usually employed only for confirmation of molluscum contagiosum infections. This can be carried out via wax sections or a squash preparation. For the squash preparation, the keratotic dome-shaped molluscum lesion is placed on a regular slide and under a coverslip or second slide. The lesion is flattened and can be examined by light microscopy directly or after staining with Wright's or methylene blue stains. Round-to-ovoid molluscum bodies up to 37 by 27 μm are diagnostic of molluscum contagiosum. Alternatively, a biopsy specimen is fixed in 10% formal saline and submitted for wax embedding, sectioning at 5 μm, and staining with hematoxylin and eosin. Microscopic examination should provide a field of view similar to that shown in Fig. 2.

EM

EM has been an important, dependable, and rapid method for diagnosis of a poxvirus infection when the results are considered along with clinical findings. Scab material or fluid from vesicles should be processed by standard methods for negative-stain EM (Nakano, 1979). When poxviruses that infect humans are viewed in this manner, the virions can be divided into two morphologically distinct groups. The first group, represented by vaccinia virus, is brick-shaped and includes the viruses variola, cowpox, vaccinia, monkeypox, tanapox, and molluscum contagiosum (size range, 140 to 230 by 210 to 380 nm) (Fig. 3A). The second group, represented by orf virus, is ovoid and elongated with criss-cross surface spicules and includes the viruses orf and pseudocowpox (milker's nodule) (size range, 120 to 160 by 250 to 310 nm). Clinically, chickenpox is the disease most often confused with monkeypox (and smallpox prior to its eradication), and EM reveals the respective virions to be quite distinct. Parapoxvirus infections can also be confidently diagnosed by EM in the context of appropriate clinical and epidemiological findings. Historically, the sensitivity of EM for detection of variola virus at the CDC was high compared to growth on CAM (98.6 and 89%, respectively) (Boulter et al., 1961). Similarly, parapoxvirus diagnosis by EM was more sensitive than tissue culture (Nakano, 1979). It is felt that the sensitivity of EM for detection of poxviruses could be close to 100% provided sufficient concentrated specimen (from pustular rash lesion) and experienced electron microscopists are available. Detection of poxvirus particles by EM does require reasonably high concentration of virions in the preparation (e.g., $>10^6$ virions/g of tissue or ml of liquid). Although EM can distinguish parapoxviruses from other poxviruses, this diagnostic method is constrained by the inability to differentiate between species within genera of poxviruses. For example, with two infections that cause similar clinical manifestations (e.g., monkeypox and variola viruses) it would be impossible to provide a virus-specific diagnosis based upon EM alone.

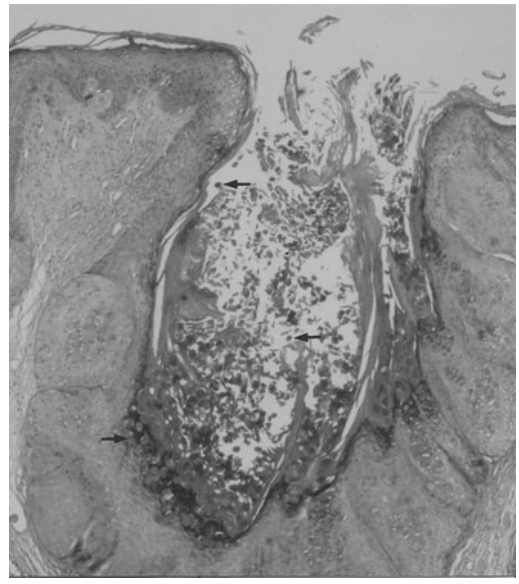

FIGURE 2 Histologic section of a molluscum contagiosum lesion. A hematoxylin- and eosin-stained wax section of a skin biopsy specimen showing hyperkeratosis and acanthosis of the epidermis. Note the hyperplasia associated with the lesion causes severe invagination of the epidermis without loss of integrity of the basal layer. Arrows indicate molluscum bodies. Magnification, ×100.

Serology

If virus lesion material is not available, then serology is an alternative approach for establishing the etiology of the disease. Historically, a large number of serologic assays have been described for measuring exposure to orthopoxviruses, parapoxviruses, and molluscum contagiosum virus, but currently only neutralization tests, Western blots, and enzyme-linked immunosorbent assays are in common use, and then only as a research tool or on a case-by-case basis at the CDC and select other institutions (Nakano, 1979; Mukinda et al., 1997; Watanabe et al., 1998; Ropp et al., 1999; Hammarlund et al., 2005; Karem et al., 2005). The use of serologic assays in the diagnosis of poxvirus infections is hampered by several factors. The existence of antibodies generated following immunization with vaccinia virus during the smallpox eradication program (as long as 30 years ago) can mask the specific detection of antibodies against other orthopoxviruses unless one employs species-specific antigen (Nakano, 1979; Ropp et al., 1999; Hammarlund et al., 2003; Hammarlund et al., 2005). Currently there are no sensitive, specific, and reliable serologic tests to retrospectively differentiate among orthopoxvirus infections. Recently, an immunoglobulin M orthopoxvirus-specific serologic assay has been validated (Karem et al., 2005), allowing determination of whether

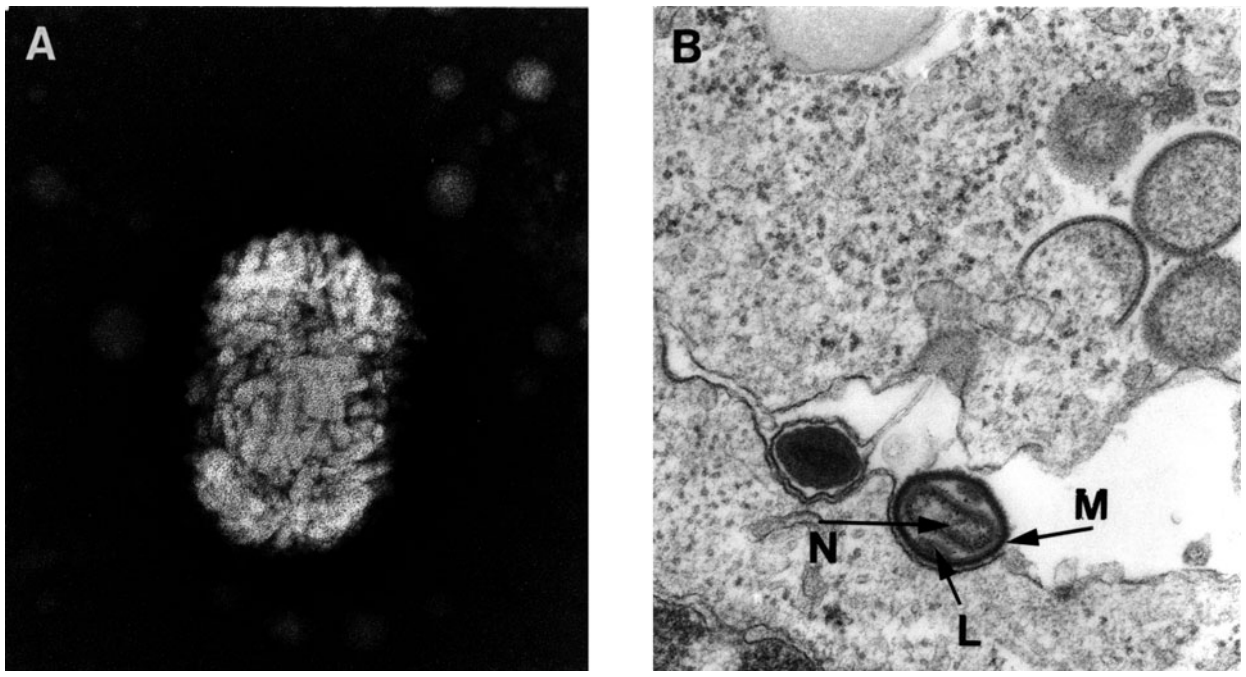

FIGURE 3 Morphology and structure of a poxvirus virion. (A) Electron micrograph of a negative-stained M form of a molluscum contagiosum virus virion. Magnification, ×120,000. Note the textured surface. (B) Electron micrograph of a thin section of a cowpox virus virion. N, nucleosome; L, lateral body; M, membrane. Note the immature forms of the virus in various stages of morphogenesis in the upper portion of the photograph. Magnification, ×120,000.

an infection was recent (estimated sensitivity from day 4 of orthopoxvirus rash to 6 months), but this test does not distinguish amongst antibodies to different species of orthopoxviruses. Following infections with parapoxvirus (such as orf and pseudocowpox [milker's nodule]) and molluscum contagiosum virus, poxvirus-specific antibodies have not always been detected or persist only transiently (Porter et al., 1992; Robinson and Lyttle, 1992; Watanabe et al., 1998; Ropp et al., 1999). Finally, disease diagnosis typically requires a fourfold rise in titer between sera drawn at the acute and convalescent phases; not all patients tolerate the inconvenience of multiple blood draws.

DNA Analysis

Characteristic genomic DNA restriction endonuclease fragment length polymorphism (RFLP) profiles are a definitive method for identification of poxvirus species; however, this approach typically requires purified viral DNA obtained after some method of replication of the virus from lesion material prior to analysis and also requires reference viral genomic DNAs for comparison. Since the advent of PCR DNA amplification, primer pairs from the gene encoding the major A-type inclusion body protein (Meyer et al., 1994) or hemagglutinin (Ropp et al., 1995) have been used to distinguish among known orthopoxvirus species. Importantly, this PCR analysis can be used directly on nucleic acid extracted from a scab or dried vesicle fluid (Ropp et al., 1995; Ropp et al., 1999; Meyer et al., 2004). Additional PCR assays have also been validated for the identification of parapoxviruses such as sealpox and orf viruses (Inoshima et al., 2000; Becher et al., 2002; Torfason and Gunadottir, 2002), the *Yatapoxvirus tanapox* (Dhar et al., 2004), and molluscum contagiosum virus (Nunez et al., 1996; Thompson, 1997; Saral et al., 2006).

Gel chromatography visualization of PCR product with or without subsequent RFLP analysis is a useful laboratory diagnostic for identification of poxviral infection to the species level but can still be relatively time-consuming (e.g., 8 to 12 h). In recent years, real-time PCR of primary lesion material has been demonstrated to provide increased diagnostic sensitivity (often down to a few genome copies), increased specificity (can differentiate a single nucleotide polymorphism), and decreased time to result. Several different technologies exist for real-time PCR, all utilizing primer pairs to amplify a short DNA sequence specific to the viral agent being studied. Most real-time PCR assays also employ a probe that, upon binding to or amplification of the target DNA sequence, releases fluorescence (Klein, 2002), allowing measurement of DNA amplification. Many of the real-time PCR assays for detection of orthopoxviral DNA (Espy et al., 2002; Sofi et al., 2003; Kulesh et al., 2004a; Kulesh et al., 2004b; Nitsche et al., 2004; Nitsche et al., 2005; Nitsche et al., 2006b; Nitsche et al., 2007; Olson et al., 2004; Panning et al., 2004; Aitichou et al., 2005; Carletti et al., 2005; Fedorko et al., 2005; Fedele et al., 2006; Li et al., 2006; Scaramozzino et al., 2007; Sias et al., 2007; Kurth et al., 2008) have been validated with a multitude of virus sample types and were instrumental in the diagnosis of monkeypox cases during the 2003 outbreak within the United States (Li et al., 2006). Similarly, real-time PCR assays for the detection of infections of molluscipoxvirus, parapoxviruses, and yatapoxviruses also have been developed (Zimmermann et al., 2005; Gallina et al., 2006; Nitsche et al., 2006a; Trama et al., 2007). The efficacy of real-time PCR assays has been

demonstrated through analysis of a variety of specimens acquired from different sources and maintained under various conditions.

Evaluation of Laboratory Diagnostic Methods

PCR RFLP and real-time PCR have been used in the rapid identification of infection by species of orthopoxvirus, parapoxvirus, molluscipoxvirus, and yatapoxvirus. These DNA-based approaches have the potential to give a definitive laboratory diagnosis based on assays with a minimal need for additional clinical or epidemiological information. The ability to determine the species of poxvirus DNA directly from lesion material without amplification of virus through either tissue culture or chicken embryo CAMs makes PCR a method of choice for rapidly detecting poxvirus DNA, with real-time PCR providing the greatest sensitivity, specificity, and speed. As with all PCR-based methods, special care is required to prevent contamination of PCRs that could result in false-positive results with serious consequences; and the presence of orthopoxvirus DNA may not necessarily be equivalent to the presence of infectious virus. Tissue culture remains the sole method for identifying whether a sample contains viable virus. EM remains a useful tool for determination of which genus (orthopoxvirus, parapoxvirus, or yatapoxvirus) of poxvirus is the cause of the infection, can be extremely quick (<30 min), and can provide an important, independent criterion for laboratory diagnosis. Although PCR RFLP and real-time PCR assays have been described for molluscum contagiosum virus (Nunez et al., 1996; Thompson, 1997; Saral et al., 2006; Trama et al., 2007), diagnosis will most likely still rely upon characteristic histopathology, since squash preparations or wax sections are both reliable and inexpensive; in fact, clinical acumen is most often used for diagnosis.

Poxvirus Diagnostics in the Future

Real-time PCR assays currently allow rapid and definitive diagnosis of the species of poxvirus causing an infection but still rely upon time-consuming processing of samples. Portable PCR machines have been designed for use in the field, requiring limited sample processing (Raja et al., 2005; Ulrich et al., 2006; Jobbagy et al., 2007; Kost et al., 2007). Robotic systems for nucleic acid extractions have become more prevalent, allowing more efficient extractions with less risk of sample contamination. Mass spectroscopy is being investigated as an adjunct to PCR for identification of amplified sequences (Hartmer et al., 2003; Tost and Gut, 2005). Future assays may rely upon pyrosequencing technologies, which can provide complete genomic sequencing, perhaps even from primary clinical material, in much less time than traditional Sanger sequencing (Margulies et al., 2005; Moore et al., 2006). Rapid sequencing not only allows for identification of the viral species but can also provide valuable subtyping information (Likos et al., 2005). Finally, possible antigen-capture methods may provide alternative protein-based approaches to poxvirus diagnostics in the future.

Conclusions

Traditionally poxvirus infections have been confirmed using a combination of laboratory tests that required the consideration of clinical and epidemiologic findings prior to a definitive diagnosis. Currently, real-time PCR technology can provide a sensitive and rapid method to detect poxvirus DNA. Cell culture remains critical for confirmation of the presence of viable virus. In the future, advances in DNA sample processing methods and pyrosequencing may increase the speed of diagnostic assays while diminishing the potential for sample contamination.

BIOLOGY OF HUMAN POXVIRUS PATHOGENS

Introduction

Classification

The earliest classification of poxviruses was based on a criterion which considered disease signs or symptoms and gross pathology. This resulted in the grouping of diseases which were characterized by pocks on the skin, including such distinct diseases as smallpox (variola virus), chickenpox (varicella-zoster virus), and syphilis (the spirochete *Treponema pallidum*). The classification of this group of disease-causing agents was refined with the application of a more-stringent criterion based on morphological characterization of virions, cytoplasmic inclusion bodies, and light microscopy (Table 1). Characteristic poxvirus elementary bodies were observed by a succession of early investigators: Chaveau in 1868, Buist in 1886, Calmette and Guerin in 1901, Prowazek in 1905, and Paschen in 1906. The infectious nature of the individual poxvirus virion was finally elucidated by Ledingham (Ledingham, 1931).

Under the auspices of a Poxvirus Subcommittee created at the Sixth International Congress for Microbiology in 1953, Fenner and Burnet (Fenner and Burnet, 1957) wrote a review which summarized the characteristics of the poxvirus group. This article remains the basis for the subsequent classification of poxviruses. The poxvirus family is divided into two subfamilies: *Entomopoxvirinae* (poxviruses of insects) and *Chordopoxvirinae* (poxviruses of vertebrates). The vertebrate poxviruses were further subclassified into genera by comparing cross-protection in animal studies, cross-neutralization of virion infectivity in cell culture, and through the analysis of genetic polymorphisms in genomic viral DNA (Table 2). The subfamily *Chordopoxvirinae* consists of 8 genera (and 6 unclassified viruses) based upon genomic comparisons: *Orthopoxvirus*, *Molluscipoxvirus*, *Parapoxvirus*, *Yatapoxvirus*, *Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, and *Suipoxvirus*. Members of the same genus have similar morphology and biological properties. Strains of the *Orthopoxvirus* genus isolated from Europe and Asia (Eurasian) are at least 94% genetically identical (Lefkowitz et al., 2006), whereas those indigenous to North America appear more divergent (Knight et al., 1992). Four genera (*Orthopoxvirus*, *Molluscipoxvirus*, *Parapoxvirus*, and *Yatapoxvirus*) are known to contain species capable of infecting humans (Table 3).

Virion Morphology and Composition

Poxvirus virions are some of the largest animal viruses and can be visualized by light microscopy when tagged, for example, with fluorescent dyes, although the details of the virion structure remain obscure. With the advent of high-resolution EM, the morphology of the poxvirus virion structure began to be revealed during the 1940s and 1950s (Dawson and McFarlane, 1948; Peters, 1956). Poxvirus virions appear to be oval or brick-shaped structures of about 200 to 400 nm in length, with axial ratios of 1.2 to 1.7 (Fig. 3A). The virion contains a noninfectious, linear, double-stranded DNA genome that varies from 130 to 300 kbp, depending on the poxvirus species, which has been reviewed in *Fields Virology*, 5th ed. (Moss, 2007). The virion has more than 100 polypeptides arranged in three structures (nucleosome

TABLE 2 Poxviruses of the vertebrates[a]

Genus	Species
Orthopoxvirus	Camelpox, cowpox, ectromelia, horsepox, monkeypox, raccoonpox, skunkpox, taterapox, Uasin Gishu, vaccinia,[b] variola, and volepox viruses
Molluscipoxvirus	Molluscum contagiosum virus[b]
Parapoxvirus	Ausdyk, bovine papular stomatitis, orf,[b] psuedocowpox, red deer pox, and sealpox viruses
Yatapoxvirus	Yaba monkey tumor[b] and tanapox viruses
Avipoxvirus	Canarypox, crowpox, fowlpox,[b] juncopox, mynahpox, pigeonpox, psittacinepox, quailpox, peacockpox, penguinpox, sparrowpox, starlingpox, and turkeypox viruses
Capripoxvirus	Goatpox, lumpy skin disease, and sheeppox[b] viruses
Leporipoxvirus	Hare fibroma, myxoma,[b] rabbit (Shope) fibroma, and squirrel fibroma viruses
Suipoxvirus	Swinepox virus[b]
Unclassified	Crocodilepox, macropod pox, Cotia pox, squirrelpox, western grey squirrelpox, and deerpox viruses

[a]Adapted from Moss, 2007, and Damon, 2007.
[b]Prototypical member.

core, lateral bodies, and membrane/envelope) (Fig. 3B), as visualized by EM of virions subjected to thin sectioning, cryosectioning, and/or negative-staining procedures. There are two forms of infectious poxvirus: the mature virion (MV), which contains a single lipid membrane bilayer surrounding the dumbbell-shaped nucleosome core and lateral bodies, and the extracellular enveloped virion (EV), which consists of the MV particle wrapped in an additional lipid membrane bilayer (Fig. 4). The envelope of MV is formed in the cytoplasmic virus factory and the virus remains cell associated unless there is cell lysis, whereas the EV receives an additional pair of membranes derived from *trans*-Golgi or endosomal cisternae, losing one of the two membranes following fusion with the plasma membrane on exiting the cell (Fig. 4).

Poxvirus Life Cycle

A Single Cycle of Virus Replication

Poxviruses are unique among DNA viruses in that, as far as is known, the entire replication cycle occurs in the cytoplasm of the host cell. The duration of the replication cycle varies greatly between poxviruses. It takes between 37 and 75 h from the time of infection to obtain maximum levels of progeny Yaba monkey tumor virus infectivity in a continuous monkey cell line (CV-1). This contrasts with a vaccinia virus replication cycle of 12 to 24 h in another continuous monkey cell line, BSC-1. The majority of information concerning the replication of poxviruses has been obtained by using vaccinia virus infections of cell lines and is depicted in Fig. 4.

Poxvirus Replication and Spread in the Host

Poxviruses infect the host mainly through the cornified epithelium of the skin or the mucosa of the respiratory tract. Infection via the skin is probably by microscopic abrasions, which allow access of the virus to the epidermal or dermal layer; all the orthopoxviruses, parapoxviruses, yatapoxviruses, and molluscum contagiosum virus can potentially infect their hosts in this manner. Epidemiological evidence strongly suggests that variola virus was primarily transmitted in excretions from the mouth or nose (and less commonly from scab material). The exact area of the respiratory tract that is initially infected, however, remains obscure (Fenner et al., 1988). It is likely that human monkeypox (monkeypox virus infection of humans) can infect via the oropharynx or nasopharynx, through abrasions of the skin, or even possibly through the oral cavity (Jezek and Fenner, 1988).

During the initial infection process, poxviruses replicate locally in epidermal cells, causing changes in the cellular structure, which can be detected with commonly used histochemical stains such as eosin and hematoxylin. Differential reactivity of these stains reveals the presence of at least three virus-specific staining patterns in infected cells. Areas of basophilic staining are referred to as B-type or Guarnieri inclusion bodies, and indicate sites of virus DNA synthesis (Guarnieri, 1892). Additionally, ectromelia virus- and cowpox virus-infected epithelial cells contain Marchal (Marchal, 1930) and Downie (Downie, 1939) bodies, respectively, which are acidophilic in character, and are now referred to as A-type inclusion bodies. A-type inclusion bodies usually contain virions embedded in a proteinaceous matrix that is mainly composed of a single polypeptide which, in the case of cowpox virus, is ~160 kDa. Although A-type inclusion bodies are not a property of all poxviruses, when they are present they are thought to prolong survival of virus in the environment. This hypothesis is supported by the apparent dependence of insect poxviruses on inclusion structures (called spherules) for dissemination and survival in nature (Bergoin and Dales, 1971). Molluscum contagiosum virus infection has its own unique intracytoplasmic, eosinophilic, granular inclusion called the molluscum body, which increases in size through the virus life cycle until the keratinocyte is devoid of any intracellular structure, except virions (Fig. 5).

Virus replicated at the site of infection spreads to the draining lymph node via the lymphatics and possibly in infected cells. At this point, poxvirus pathogenesis follows one of two courses. Some viruses cause either a localized, self-limited infection with little spread from the original site of inoculation; such is the case with molluscum contagiosum virus, Shope fibroma virus, or Yaba monkey tumor virus. Others cause a fulminant, systemic infection characterized by a generalized rash and high mortality rate, as with variola virus, human monkeypox, or ectromelia virus infections of mice. In certain cases, both of these disease patterns can be caused by the same virus but in different host species. Classic examples are infections by cowpox virus and myxoma virus. In humans and cows, cowpox virus causes a localized pustular skin lesion, whereas generalized infections with

TABLE 3 Genera of vertebrate poxviruses which infect humans[a]

Genus	Species	Geographic distribution	Reservoir host	Other known infected hosts
Orthopoxvirus				
Eurasian	Camelpox virus[b]	Africa, Asia	Camels	None
	Cowpox virus[b]	Europe, western Asia	Bank voles, long-tailed field mice	Cats, cattle, **humans,** zoo animals
	Ectromelia virus[b]	Europe	Rodents	None
	Horsepox virus[b]	Central Asia	Unknown	Horses
	Monkeypox virus[b]	Western and central Africa	Unknown (likely rodents)	Monkeys, zoo animals, **humans,** prairie dogs, rodents
	Taterapox virus[b]	Western Africa	Gerbils	None
	Uasin Gishu virus	Eastern Africa	Unknown	Horses
	Vaccinia virus[b]	Unknown	Unknown	**Humans,** rabbits, cows, river buffaloes
	Variola virus[b]	Eradicated (formerly worldwide)	**Humans**	None
North American	Raccoonpox virus	Eastern United States	Raccoons	None
	Skunkpox virus	Western United States	Skunks	None
	Volepox virus[b]	Western United States	California voles	None
Molluscipoxvirus	Molluscum contagiosum virus[b]	Worldwide	**Humans**	None
Parapoxvirus	Ausdyk virus	Africa, Asia	Camels	None
	Bovine popular stomatitis virus[b]	Worldwide	Cattle (beef)	**Humans**
	Orf virus[b]	Worldwide	Sheep, goats	Ruminants, **humans**
	Pseudocowpox virus	Worldwide	Cattle (dairy)	**Humans**
	Red deer poxvirus	New Zealand	Red deer	None
	Sealpox virus	Worldwide	Seals	**Humans**
Yatapoxvirus	Tanapox virus	Eastern and central Africa	Unknown	**Humans**
	Yaba monkey tumor virus[b]	Western Africa	Unknown (likely primates)	**Humans**

[a]Host range and features of members of the subfamily *Chordoppoxvirinae* that contain species which cause human disease. Adapted from Damon, 2007, and Moss, 2007.
[b]Completely sequenced.

pustular rashes are observed in many species from the family Felidae. Myxoma virus produces localized benign fibromas in wild rabbits of North and South America, whereas the infection of the European rabbit results in a generalized infection with a high mortality rate.

In systemic infections (based on the mousepox [ectromelia virus] model) (Fenner, 1948), virus passes out of the draining lymph node via the efferent lymphatics and enters the venous system through the thoracic ducts. This primary viremia results in the seeding of virus into the liver, spleen and/or other organs, with the target organs and/or cells differing among the poxviruses. For example, ectromelia virus replicates to high levels in the fibroreticular cells but not the lymphocytes of the reticuloendothelial system, whereas myxoma virus replicates well in the lymphocytes (McFadden, 1988; Karupiah et al., 1993). Further virus replication in these internal organs liberates large quantities of virus into the bloodstream, and this secondary viremia results in productive infections of kidneys, lungs, intestines, skin, and/or other organs. Infection of the skin can give rise to maculopapular then vesiculopustular rash.

Transmission of virus between an index case and a susceptible host can originate from the primary site of infection, which is most likely the case in localized self-limited infections such as molluscum contagiosum, or from virus produced in the "end-organ" epithelia as a result of the secondary viremia. In the case of smallpox, variola virus was released from lesions in the mouth, nose, and pharynx into the nasal and oropharyngeal secretions during the first week of the rash.

Recovery from poxvirus infections involves both innate immune mechanisms (e.g., interferon, complement, and natural killer cells) and adaptive responses (e.g., delayed-type hypersensitivity, cytotoxic T lymphocytes, and specific antibody alone and in combination with natural killer cells) (Buller and Palumbo, 1991; Mahalingam et al., 2000). Of these host responses, cell-mediated T-cell responses are thought to be most important, although the complete repertoire of the host defense armamentarium is likely required for recovery from infection with a highly virulent poxvirus such as variola. Recent studies of ectromelia, within the mouse model, confirm that T-cell responses are important for protection

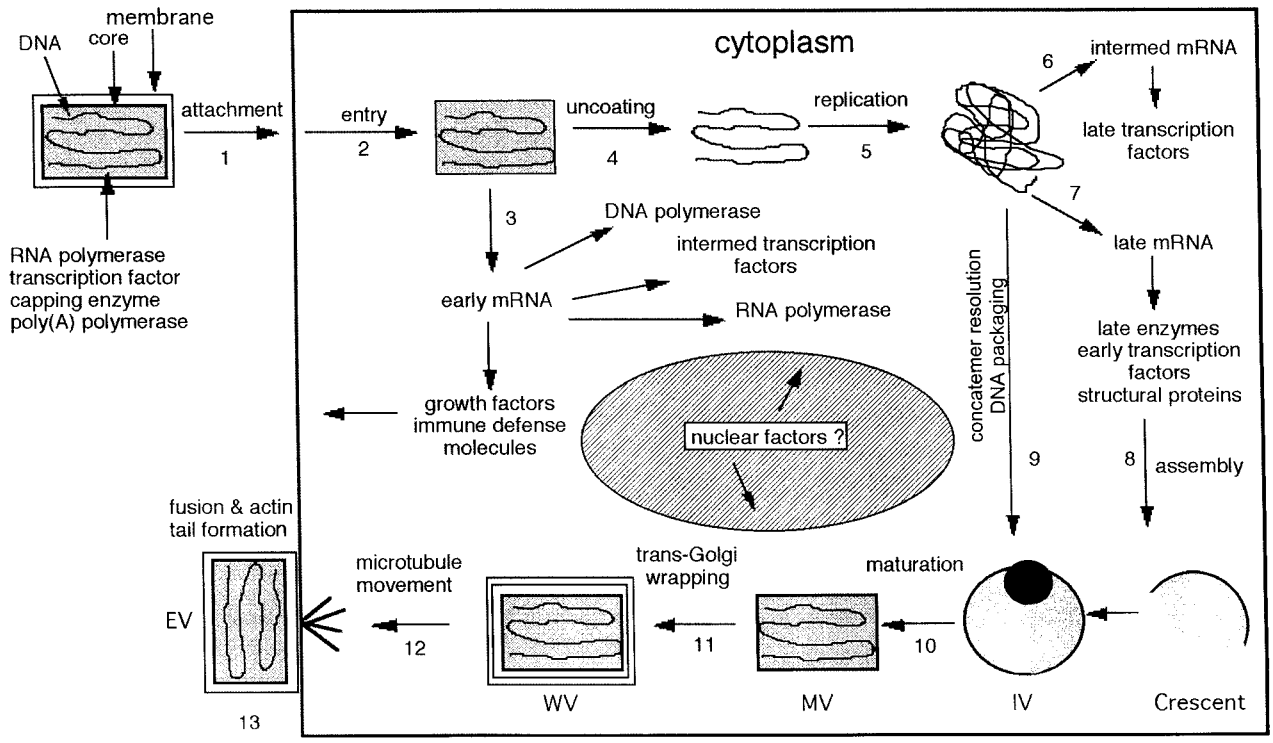

FIGURE 4 Replication cycle of vaccinia virus. A virion, containing a double-stranded DNA genome, enzymes, and transcription factors, attaches to a cell (1) and fuses with the cell membrane, releasing a core into the cytoplasm (2). The core synthesizes early mRNA that is translated into a variety of proteins, including growth factors, immune defense molecules, enzymes, and factors for DNA replication and intermediate transcription (3). Uncoating occurs (4), and the DNA is replicated to form concatemeric molecules (5). Intermediate genes in the progeny DNA are transcribed, and the mRNA is translated to form late transcription factors (6). The late genes are transcribed, and the mRNA is translated to form virion structural proteins, enzymes, and early transcription factors (7). Assembly begins with the formation of discrete membrane structures (8). The concatemeric DNA intermediates are resolved into unit genomes and packaged in immature virions (IV) (9). Maturation proceeds to the formation of infectious intracellular MV (10). The MVs are wrapped by modified *trans*-Golgi and endosomal cisternae (11), and the wrapped virions (WV) are transported to the periphery of the cell along microtubules (12). Fusion of the WVs with the plasma membrane results in release of extracellular EV (13). The actin tail polymerizes in the cytoplasm beneath the EV (13). Although replication occurs entirely in the cytoplasm, nuclear and cytoplasmic cell factors may be involved in transcription and assembly. Reprinted from *Fields Virology*, 5th ed. (D. M. Knipe and P. M. Howley [ed.], Lippincott Williams & Wilkins [Moss, 2007]).

from primary infection but demonstrated that for protection from secondary ectromelia infection, antibody is absolutely required (Panchanathan et al., 2008). In evaluation of vaccine-related protection, the humoral response appears critical. Upon recovery from infection, the duration of poxvirus immunity varies dependent upon the genus: lifelong protection after recovery from smallpox or transient protection with orf.

POXVIRUSES PATHOGENIC FOR HUMANS

Eleven poxviruses have been documented to infect humans (Table 3) (Damon, 2007). Except for the "extinct" variola and molluscum contagiousum virus, the other poxvirus diseases are zoonoses. With the exception of monkeypox virus, these zoonotic poxviruses fail to maintain human-to-human transmission. Most human poxvirus infections occur through minor abrasions in the skin. Orf and molluscum contagiosum viruses cause the most frequent poxvirus infections worldwide with the incidence of molluscum contagiosum virus on the rise, especially as an opportunistic infection of late-stage AIDS patients (Schwartz and Myskowski, 1992a). Indeed, as populations of immune-impaired patients increase, the frequency of all human poxvirus-induced disease may rise.

Orthopoxviruses

Four orthopoxviruses have been shown to cause disease in humans. The most notorious was smallpox, a solely human pathogen, whose date of emergence as a human pathogen remains uncertain (Li et al., 2007). In 1967, two clinically distinct types of smallpox, referred to as variola major and variola minor, remained endemic in 33 countries. Variola major occurred primarily in Asia and caused the most severe disease, with aggregate case fatality rates up to 30%. Variola minor was associated with case fatality rates between 0.1 and 2%. Retrospective genomic analysis of 45 variola isolates has segregated strains into phylogenetic subgroups, which correlate with geographic origin and reported case fatality rates (Esposito et al., 2006; Li et al., 2007). Since the global eradication of smallpox in 1979, variola virus has no longer circulated in nature (Fenner et al., 1988). At this

FIGURE 5 Electron micrograph of a thin section of a molluscum contagiosum virus-infected cell or molluscum body. All of the cellular organelles are beyond recognition, having been pushed to the periphery of the cell by the masses of virions. Magnification, ×3,000.

time, all known stocks of variola virus are held in two WHO collaborating centers: the U.S. CDC in Atlanta, GA, and the State Center of Virology and Biotechnology (VECTOR) in Kotsovo, Russia. Although the WHO Ad Hoc Committee on Orthopoxvirus Infections has recommended the destruction of the remaining stocks of variola virus, this course of action has been delayed pending the assessment of future scientific needs for live variola virus, as noted previously.

Eradication of smallpox was made possible by the availability of a live vaccine and at least three characteristics of variola virus: one stable serotype, virus transmission only after a relatively long incubation period and prodrome, and no animal reservoir of the virus. Smallpox vaccine was another orthopoxvirus, vaccinia; a historic vaccine with an unknown pedigree. Vaccinia virus did not provide sterilizing immunity but did protect vaccinees from severe disease following a subsequent infection with variola virus. Vaccine preparations of unattenuated vaccinia virus, however, are not completely avirulent and have caused vaccine-related complications, especially in immune-impaired individuals (e.g., accidental infection, generalized vaccinia, eczema vaccinatum, erythema multiforme, progressive vaccinia, and postvaccinal encephalitis; 1,253 cases/1 million primary vaccinations) (Lane et al., 1969; Buller and Palumbo, 1991). With the eradication of variola virus, the need for continued immunization was diminished, and by March of 1983, the WHO reported that >96% of the 160 member states had officially discontinued immunization. Currently, smallpox vaccination of humans in the United States is limited to the controlled immunization of personnel that handle orthopoxviruses capable of infecting humans in the laboratory, the military, and select healthcare workers (Rotz et al., 2001; Wharton et al., 2003). It is recommended that laboratory workers who directly handle cultures, animals, and/or materials contaminated or infected with non-highly attenuated vaccinia and their recombinants or other orthopoxviruses capable of infecting humans (e.g., monkeypox, cowpox, and others) be immunized with vaccinia virus vaccine. Immunization is not recommended for persons who do not directly handle virus cultures or who do not work with materials contaminated or animals infected with these viruses. Reimmunization should be carried out according to the Advisory Committee on Immunization Practices (ACIP) recommendations. Administering physicians should contact the CDC Drug Service and the CDC National Immunization Program for details on obtaining smallpox (vaccinia virus) vaccine and for advice on clinical questions (Rotz et al., 2001).

Two examples of emerging orthopoxvirus zoonoses are postulated to be due to vaccinia virus, used by the WHO during the smallpox eradication campaign, and occur in domesticated animals and possible wild animal reservoirs. Buffalopox was documented in the 1960s and 1970s in herds of milking buffalos and dairy cattle in Asia and Africa (Kolhapure et al., 1997). Cantagalo, Aracatuba, and other vaccinia-like viruses may similarly have originated from the "escape" of vaccinia virus from the WHO smallpox eradication campaign in Brazil (1967 to 1979) into cattle herds (Damaso et al., 2000; da Fonseca et al., 2002; de Souza et al., 2003; Nagasse-Sugahara et al., 2004; Trindade et al., 2006; Trindade et al., 2007). Alternatively, these viruses may be more directly genetically related to the "original" vaccinia virus, the exact origin of which remains obscure. These agents appear to be transmitted to humans by direct contact with infected bovids; lesions are confined primarily to the fingers, hands, and face, and morbidity has been described. No generalized human infection has been documented. Limited person-to-person transmission may occur.

Currently only monkeypox, and perhaps cowpox and vaccinia-like viruses, cause significant human infections in immunocompetent hosts. Cowpox viruses cause a disease presentation similar to that of the emerging vaccinia-like viruses. Disseminated cowpox occurs rarely (Blackford et al., 1993), and fatalities have been reported (Czerny et al., 1991). Since the eradication of smallpox, monkeypox is typically the most severe orthopoxvirus disease in humans. Clinical signs of human monkeypox were difficult to distinguish from smallpox; the primary distinctive feature was pronounced lymphadenopathy seen in monkeypox patients. Fortunately, monkeypox is less efficiently spread from human to human and has a lower case fatality rate than smallpox (Jezek and Fenner, 1988). Although monkeypox is only endemic within Africa, the 2003 outbreak of human monkeypox within the United States, due to importation of African rodents (Reed et al., 2004), reinforces the need to study and recognize orthopoxviruses as emerging infectious diseases and demonstrates the potential for international dispersal of zoonotic orthopoxviruses.

Pathogenesis

Transmission

The exact mode of transmission of monkeypox virus from a nonhuman animal source to humans is not known but may be via the oropharynx or nasopharynx or through abrasions of the skin (as perhaps during butchering of nonhuman species). Person-to-person transmission (as was seen with smallpox) is believed to be by the upper respiratory tract, with virus released in oropharyngeal secretions of patients who have a rash (Jezek and Fenner, 1988). Unlike smallpox, monkeypox person-to-person transmission is less efficient ($R_{oSmallpox}$ = 3.5 to 6 versus $R_{oMonkeypox}$ = 0.8) (Fine et al., 1988;

Gani and Leach, 2001); however, recently an extended chain of six generations of confirmed human-to-human transmission was documented (Learned et al., 2005).

Cowpox virus is usually acquired by direct introduction of the virus from a nonhuman animal source into minor abrasions in the skin; however, 30% of human infections have no known risk factor for infection or obvious route of inoculation (Baxby et al., 1994). Similarly, vaccinia virus and "vaccinia-like" virus infections are usually due to close contact with a recent smallpox (vaccinia) vaccinee or infected cattle, respectively (Damaso et al., 2000; da Fonseca et al., 2002; Nagasse-Sugahara et al., 2004).

Lesion Histopathology

Orthopoxvirus lesions are characterized with epidermal hyperplasia; with infected cells becoming swollen and vacuolated and undergoing "ballooning degeneration." The cells contain the irregular, faint, B-type inclusion bodies. Cowpox skin lesions from nonhuman animals contain A-type inclusion bodies in epidermal cells, sebaceous glands, and endothelial cells; however, similar inclusion bodies are not observed in the few human cowpox lesions examined (Baxby et al., 1994).

Epidemiology

Geographical Distribution

Monkeypox viruses are found in the tropical rain forests of countries in western and central Africa. There are two clades of monkeypox virus (Likos et al., 2005), with the less virulent strains occurring within West Africa. The Congo Basin clade has higher reported case fatality rates (up to 12%) and is found most notably in the Democratic Republic of the Congo (formerly Zaire). Human-to-human transmission has only been documented with virus from the Congo Basin monkeypox virus clade. The reservoir of monkeypox virus is unknown but is most likely one or more rodent species. Viable monkeypox virus has been isolated from three African species imported into the United States: giant pouched rats (*Cricetomys* sp.), rope squirrels (*Funisciurus* sp.), and dormice (*Graphiuris* sp.) (Hutson et al., 2007). In fact, only once has viable monkeypox virus been recovered in African wildlife, from a moribund squirrel discovered in Zaire (Khodakevich et al., 1986).

Cowpox viruses are endemic to Europe and some western states of the former Soviet Union. Rodents (voles, wood mice, and rats) have been implicated as reservoirs of cowpox viruses in Great Britain; with humans, cows, zoo animals, and cats as incidental hosts (Baxby et al., 1994; Hazel et al., 2000). Vaccinia-like viruses are known to be endemic to Brazil and India, found in dairy cattle and buffalo herds (Kolhapure et al., 1997; Damaso et al., 2000; da Fonseca et al., 2002; de Souza et al., 2003; Nagasse-Sugahara et al., 2004; Trindade et al., 2006; Trindade et al., 2007); the possible wild animal reservoirs of these viruses are unknown.

Prevalence and Incidence

WHO intensive surveillance for human monkeypox between 1981 and 1986 in Zaire confirmed 65 cases annually with the greatest risk of infection to inhabitants of small villages within 100 m of tropical rain forests (Jezek and Fenner, 1988). Recently, monkeypox has reemerged on a scale of greater magnitude than previously seen. Between February 1996 and October 1997, 250 suspect cases of human monkeypox were identified in a population of 500,000. Approximately 75% of the cases appeared to be due to human-to-human transmission, although the secondary attack rate was ~8% among unvaccinated contacts, which is similar to the historical value (Centers for Disease Control and Prevention, 1997a, 1997b; Mukinda et al., 1997; World Health Organization, 1997a, 1997b). Reports from the Democratic Republic of the Congo in 2001 to 2004 document 136 suspect monkeypox cases, 51 of which were confirmed as monkeypox by PCR (Rimoin et al., 2007). The reemergence of monkeypox may be due to waning immunity following cessation of the smallpox immunization program, increased encroachment of larger human populations into the primary habitat of the animal reservoir of the virus, heightened surveillance, or a combination of these factors and possibly others. Furthermore, monkeypox is an emerging zoonosis of potential concern worldwide. Importation of African rodents led to an outbreak of human monkeypox within the United States in 2003 (Centers for Disease Control and Prevention, 2003; Reed et al., 2004), reinforcing the concept that poxviruses can be encountered outside their normal geographic range and may pose a serious threat of becoming established as agents of persistent zoonotic disease in novel ecologies.

The majority of human cowpox transmission is by the domestic cat, although human infections have been acquired from cows and rodents. Between 1969 and 1993, there were approximately 45 human cowpox cases in Britain, three published case histories from Germany, and two each from Belgium, Sweden, and France (Baxby et al., 1994). Vaccinia-like virus infections, associated with bovine contact, have been increasingly reported in Brazil (Damaso et al., 2000; da Fonseca et al., 2002; de Souza et al., 2003; Nagasse-Sugahara et al., 2004; Trindade et al., 2006; Trindade et al., 2007).

Diagnosis

Clinical Signs, Symptoms, and Severity

Approximately 12 days after infection with monkeypox virus, fever and headache occur. This is followed 1 to 3 days later by a rash and generalized lymphadenopathy. The rash (the number of lesions is variable) typically appears first on the face and generally has a centrifugal distribution (Fig. 6). The illness lasts 2 to 4 weeks depending on its severity. The case fatality rate is up to 12% for the Congo Basin clade and less than 1% for the West African clade (Jezek and Fenner, 1988; Likos et al., 2005).

With human cowpox virus infection, a lesion, usually solitary, appears on the hands or face. This can be extremely painful, and the patient can present with systemic symptoms, including pyrexia, malaise, lethargy, sore throat, and local lymphadenopathy. Complete recovery takes between 3 and 8 weeks. Person-to-person transmission has not been reported. Complications can include ocular or generalized infections; the latter occur in patients with atopic dermatitis, allergic asthma, or atopic eczema and, in one case, was associated with death (Baxby et al., 1994). Similarly, vaccinia-like viruses cause painful localized lesions usually on the hands or arms (Damaso et al., 2000; da Fonseca et al., 2002; de Souza et al., 2003; Nagasse-Sugahara et al., 2004; Trindade et al., 2006).

Gross Lesion Pathology

The monkeypox virus skin lesions begin as macules which progress over the course of days from papules to vesicles to pustules. At about 8 or 9 days after the onset of rash, the pustules become umbilicated and dry up; by 14 to 16 days after the onset of the rash, a crust has formed. Most skin lesions are about 0.5 cm in diameter (Jezek and Fenner, 1988).

FIGURE 6 Monkeypox rash. A 7-year-old Zairian girl is shown 2 days after the onset of the rash. Courtesy of M. Szczeniowski.

The cowpox lesion appears as an inflamed macule and progresses through an increasingly hemorrhagic vesicle stage to a pustule which ulcerates and crusts over by the end of the second week, becoming a deep-seated, hard black eschar 1 to 3 cm in diameter (Fig. 7) (Baxby et al., 1994). Vaccinia-like viruses cause a similar lesion, usually without the hemorrhagic manifestation (Fig. 8) (Damaso et al., 2000; da Fonseca et al., 2002; de Souza et al., 2003; Nagasse-Sugahara et al., 2004; Trindade et al., 2006).

Diagnosis and Differential Diagnoses

Previously, the diagnosis of monkeypox typically required clinical (rash), epidemiologic (equatorial Africa), and laboratory (brick-shaped virion in scab material and/or orthopoxvirus antigen, nucleic acid, or virus detected in lesions) findings. Although the rash with associated lymphadenopathy is usually pathognomonic, the sporadic nature of the disease contributed to possible failure to arrive at an accurate diagnosis solely on clinical grounds (Jezek and Fenner, 1988). The cocirculation of varicella virus (including cases that present with potentially atypical clinical signs) and monkeypox virus in Africa continues to present challenges to definitive clinical diagnosis. Furthermore, the increased spread of human monkeypox to new geographic locales complicates epidemiologic analysis (Reed et al., 2004; Damon et al., 2006). Due to its clinical similarity to smallpox and the increasing concern over potential bioterrorism, quick identification of monkeypox virus infection is critical to an effective public health response. Laboratory diagnostic assays (real-time PCR) are now strongly relied upon for rapid identification of human monkeypox cases.

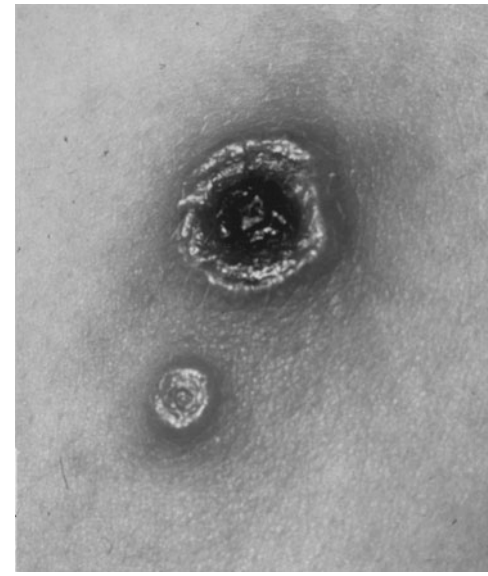

FIGURE 7 Primary and secondary lesions of cowpox. The primary lesion is at the early eschar stage (probably 2 to 3 weeks after infection), whereas the secondary lesion (below) is at the early vesicular stage. Provided by M. White; reprinted with permission from Baxby et al., 1994.

Monkeypox can be confused with a number of other conditions that result in a rash, including the following: chickenpox, although its varicella-zoster lesions are more superficial, usually irregularly bordered, appear in crops, with individual lesions evolving quickly (days), and have a centripetal

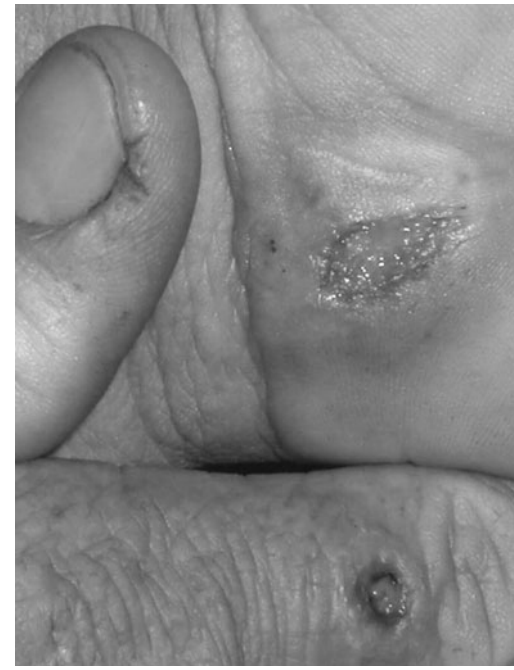

FIGURE 8 Vaccinia-like lesions. Lesions found on the hands of a milker within Minas Gerais State, Brazil. Courtesy of Bovine Vaccinia Investigation Group, Federal University of Minas Gerais State, Brazil.

distribution; tanapox, although tanapox lesions evolve slowly (weeks) and are nodular and large in size without pustulation; and syphilis, although the secondary rash of syphilis does not evolve past the papular stage (Jezek and Fenner, 1988).

Cowpox diagnosis is rarely made based on clinical findings (lesion morphology and systemic illness) and usually requires laboratory results (brick-shaped virion or presence of cowpox DNA in scab or lesion material). Cowpox should be considered in patients (especially Europeans) who have had contact with cats and who present in July to October with a painful hemorrhagic vesicle or black eschar, with or without erythema, accompanied by lymphadenopathy and a systemic illness (Baxby et al., 1994).

Generalized cowpox can be misdiagnosed as eczema herpeticum, whereas localized cowpox is most frequently misdiagnosed as orf or pseudocowpox (milker's nodule), although the parapoxvirus lesion is clinically distinct, usually not painful, and often has no systemic signs or symptoms; herpesvirus reactivation, although herpes lesions are not usually hemorrhagic or erythematous and the scab is not as deep-seated and is of lighter color; anthrax, although anthrax lesions are painless and rapidly progress to the eschar stage (5 to 6 days) (Baxby et al., 1994).

Vaccinia virus infections are usually identified based on epidemiologic (recent contact with smallpox [vaccinia] vaccinees or bovids within specific geographic locales) and laboratory findings (brick-shaped virion or presence of vaccinia DNA in scab or lesion material). Clinical confusion often occurs with similar etiologic agents as listed above for cowpox.

Molluscum Contagiosum Virus

Pathogenesis

Transmission

Molluscum contagiosum was first clinically recognized in 1817 (Snell and Fox, 1961). The incubation period of molluscum contagiosum is poorly understood, but epidemiologic studies suggest it ranges from weeks to months. Molluscum contagiosum is observed in children and adults, with spread within this latter group governed in part by sexual practices. Nonsexual transmission is a consequence of infection by direct contact or through fomites. For example, case histories have suggested transmission from surgeons' fingers, behaviors associated with swimming pools, bath towels in gymnasia, contact between wrestlers, and the tattooing process (Postlethwaite, 1970). Transmission between persons in the absence of fomites requires intimate contact. Lesions can be commonly observed on opposing epithelial surfaces and the virus can be further spread by autoinoculation.

Lesion Histopathology

Epidermis. Molluscum contagiosum virus has one of the most limited host cell tropisms of any virus, replicating only in the human keratinocyte of the epidermis (Buller et al., 1995). Molluscum contagiosum virus replicates in infected keratinocytes in the lower layers of the stratum spinosum, and lesions become more prominent during the 9 to 15 days it takes the infected keratinocyte to reach the stratum granulosum. As the progeny virions accumulate within the granular cytoplasmic matrix, the cellular organelles, including the nucleus, are forced to the periphery of the cell and contribute to the overall increase in cell size. By light microscopy, these cells stain as hyaline acidophilic masses, are referred to as molluscum or Henderson-Patterson bodies, and are pathognomonic for disease (Fig. 2). Higher magnification of the molluscum body reveals a cell almost entirely filled with virions (Fig. 5). As a consequence of these hypertrophied, infected cells and hyperplasia of the basal cells, the molluscum lesion extends above the adjacent skin as a tumor and projects down into the dermis without breaking the basement membrane.

Dermis. In lesions in which the basement membrane is intact, the dermis is usually not involved.

Epidemiology

Geographic Distribution

Molluscum contagiosum virus has a worldwide distribution but is more prevalent in the tropics. Analysis of genomic DNA from molluscum contagiosum virus isolates has revealed the existence of at least four virus subtypes. Several studies suggest the distribution of subtypes can vary geographically (Porter and Archard, 1992; Nakamura et al., 1995; Agromayor et al., 2002; Saral et al., 2006).

Prevalence and Incidence

For non-sexually transmitted molluscum contagiosum, the disease is more prevalent in the tropics. For example, molluscum contagiosum was diagnosed in 1.2% of outpatients in Aberdeen, Scotland between 1956 and 1963, the mean age of infection was between 10 and 12 years old, and spread within households and schools was infrequent. On the other hand, in Fiji in 1966, 4.5% of an entire village had the disease, the mean age of infection was between 2 and 3 years old, and 25% of households harbored more than one case (Postlethwaite et al., 1967; Postlethwaite, 1970; Porter et al., 1992). In England between 1971 and 1985, there was a 400% increase in cases of genital molluscum contagiosum, with the majority of the cases in the 15- to 24-year-old group (Porter et al., 1992). In the United States between 1966 and 1983, there was a 10-fold increase in cases in patients aged 25 to 29 years (Porter et al., 1992). Before effective retroviral therapies, molluscum contagiosum was a common and sometimes severely disfiguring opportunistic infection of human immunodeficiency virus (HIV)-infected patients (5 to 18%), especially those with severely depressed $CD4^+$-T-cell numbers (Schwartz and Myskowski, 1992b).

Diagnosis

Clinical Signs, Symptoms, and Severity

Clinically, molluscum contagiosum presents as single or small clusters of lesions in immunocompetent individuals. There are no other signs or symptoms associated with the disease. The lesions are generally painless, appearing on the trunk and limbs (except palms and soles) in the non-sexually transmitted disease. In children, disease can also be fairly common in the skin of the eyelids, with solitary or multiple lesions, and can be complicated by chronic follicular conjunctivitis and later by a superficial punctate keratitis (al-Hazzaa and Hidayat, 1993). There may be an associated erythema 1 to 11 months following the appearance of the lesion with no correlation to a history of allergy or eczema (Binkley et al., 1956). As a sexually transmitted disease in teenagers and adults, the lesions are mostly on the lower abdominal wall, pubis, inner thighs, and genitalia. Lesions can persist for as little as 2 weeks or as long as 2 years. Reinfections can be common. Although there may be an association between virion genomic subtype and geographic location

(Porter and Archard, 1992; Nakamura et al., 1995; Agromayor et al., 2002; Saral et al., 2006), as yet there is no solid correlation of virion DNA subtype with specific pathology or location on the body (e.g., genital versus nongenital lesions) (Thompson et al., 1990).

In immunocompromised individuals (especially in persons with AIDS), molluscum contagiosum is typically not self-limiting and more and larger lesions are observed, especially on the face, neck, scalp, and upper body, with multiple adjacent lesions sometimes becoming confluent. Molluscum contagiosum can be considered a cutaneous marker of severe immunodeficiency.

Gross Lesion Pathology

In immunocompetent patients, the molluscum contagiosum lesions begin as pimples and become umbilicated, epidermal, flesh-colored, raised nodules of 2 to 5 mm in diameter. A semisolid caseous material can be expressed from the center of the lesion, is rich in molluscum bodies, and probably is responsible for disease transmission. Rarely, the disease will present as a large lesion (>5 mm in diameter) called "giant molluscum" (Fig. 9). Giant molluscum lesions (>5 mm) have been reported more frequently in severely immunodeficient HIV-infected individuals (Schwartz and Myskowski, 1992b; Izu et al., 1994), although the large lesions were observed in New Guinea prior to the introduction of HIV, and therefore were not solely a consequence of HIV-induced immunodeficiency.

Diagnosis and Differential Diagnoses

The diagnosis of molluscum contagiosum is usually made clinically based on gross appearance of the lesions and their chronic nature. Laboratory confirmation is easily obtained by hematoxylin and eosin staining of a biopsy specimen or by a squash preparation of expressed material from the lesion (Fig. 2). Several PCR RFLP and real-time PCR assays have been described for molluscum contagiosum virus (Nunez et al., 1996; Thompson, 1997; Saral et al., 2006; Trama et al., 2007) which allow for subtyping when indicated.

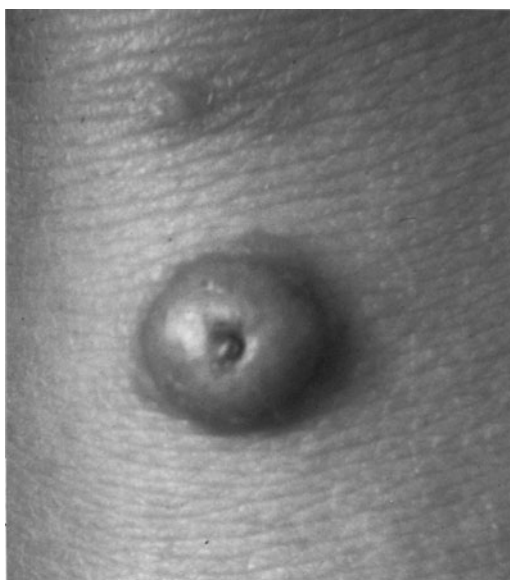

FIGURE 9 Molluscum contagiosum lesions. A giant molluscum lesion next to a more typical lesion. Note the umbilicated center. Courtesy of J. Burnett.

Molluscum contagiosum (especially giant molluscum) can be confused with a number of other disorders such as keratoacanthoma, warty dyskeratoma, syringomas, hidrocystomas, basal cell epithelioma, trichoepithelioma, ectopic sebaceous glands, giant condylomata acuminata, chalazion, sebaceous cysts, verrucae, and milia or granuloma on eyelids (Janniger and Schwartz, 1993; Itin and Gilli, 1994; O'Neil and Hansen, 1995). In immunodeficient patients, disseminated cutaneous cryptococcosis and histoplasmosis may resemble molluscum contagiosum (Janniger and Schwartz, 1993). An inflamed molluscum lesion without the association of typical lesions may be mistaken for a bacterial infection.

Parapoxviruses

The parapoxviruses, including orf, bovine papular stomatitis, pseudocowpox (milker's nodule), and sealpox viruses cause occupational infections of humans, with orf infections being the most common. Wildlife (skinning animals such as deer and reindeer) also have been sources of parapoxvirus infection. The majority of human parapoxvirus infections probably go unreported, as many sheep farmers and rural physicians are aware of the diseases and make a diagnosis based solely on clinical findings and do not seek treatment. No human-to-human transmission of parapoxvirus infections has been reported.

Pathogenesis

Transmission

Direct transmission of orf virus has been observed as a consequence of bottle-feeding lambs, animal bites to the hand, and contact with sheep and goat products during slaughter. Fomites found on items such as wooden splinters, barbed wire, or farmyard surfaces such as soil, feeding troughs, or barn beams have been implicated as sources for possible virus inoculation. No human-to-human transmission of orf virus has been reported.

Bovine papular stomatitis virus infections of humans are thought to occur from contact with lesions found on the mouth, tongue, lips, or nares and occasionally the teats of infected cattle.

Pseudocowpox virus from lesions on teats of cows is a major source of virus infection for milker's nodule of the hand.

Persons in direct physical contact with pinnipeds have reported an "orf-like" lesion after being bitten by infected harbor seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) (Wilson et al., 1972a, 1972b; Hicks and Worthy, 1987). After the advent of reliable PCR tests for parapoxviruses (Becher et al., 2002), sealpox virus was confirmed as the causative agent of the orf-like lesion (Clark et al., 2005).

Lesion Histopathology

Histopathological features of human orf and pseudocowpox lesions are indistinguishable and are similar to human lesions caused by bovine papular stomatitis virus and sealpox virus. For this reason, and since orf infections are the most common human parapoxvirus infections, only the histopathological features of human orf will be presented (Groves et al., 1991).

Epidermis. The most striking change in the epidermis during orf virus infections is hyperplasia in which strands of epidermal keratinocytes penetrate into the dermis. Generally, a mild-to-moderate degree of acanthosis is detected, and parakeratosis is a common feature. Cytoplasmic vacuolation,

nuclear vacuolation, and deeply eosinophilic, homogeneous cytoplasmic inclusion bodies, often surrounded by a pale halo, are also characteristic of the infection. An intense infiltration of lymphocytes, polymorphonuclear leukocytes, or eosinophils frequently involves the epidermis.

Dermis. A dense, predominantly lymphohistiocytic inflammatory cell infiltrate is present in all orf cases. Also there is marked edema both vertically and horizontally that may contribute to the overall papillomatous appearance. The most striking feature of the infected dermis is the massive capillary proliferation and dilation. The former is thought to be the result of the action of a virus-encoded growth factor which has homology to mammalian vascular endothelial growth factor (Lyttle et al., 1994).

Epidemiology

Geographic Distribution

Orf in sheep and goat populations has been reported in Canada, the United States, Europe, Japan, New Zealand, and Africa. Pseudocowpox virus occurs in dairy herds of European-derived cattle found in all parts of the world. Bovine papular stomatitis virus is similarly distributed but is found more often associated with beef rather than dairy animals. Seal and other pinniped populations worldwide have been found to harbor sealpox virus.

Prevalence and Incidence

In a 1-year New Zealand study, 500 meat workers from an at-risk population of 20,000 were infected with orf, with the highest risk (4%) of infection for those involved in the initial butchering of the sheep (Robinson and Petersen, 1983). Serologic surveys of orf-infected sheep and goat herds yielded orf antibody prevalences of up to 90%. The high seroprevalence of orf antibody in herds is believed to be associated with the highly stable nature of the orf virion, which contaminates the environment and causes reintroduction (Mercer et al., 1997; Lederman et al., 2007a).

Pseudocowpox virus has been found to be endemic in cattle herds in West Dorset, England (Nagington et al., 1965). Pseudocowpox and bovine papular stomatitis viruses are probably endemic in all European-derived dairy herds. Sealpox virus was identified as a unique species of parapoxvirus in 2002 (Becher et al., 2002), but disease has been seen in pinnipeds found in Europe and North America since 1969 (Wilson et al., 1969).

Diagnosis

Clinical Signs, Symptoms, and Severity

The clinical presentation of orf usually occurs 3 to 4 weeks postinfection. The human disease involves the appearance of single or multiple nodules (diameters of 6 to 27 mm) (Groves et al., 1991), which are sometimes painful, usually on the hands, and less frequently on the head or neck. Orf infection can also be associated with a low grade fever, swelling of the regional lymph node, and/or erythema multiforme bullosum. Resolution of the disease occurs over a period of 4 to 6 weeks, usually without complication; however, autoinoculation of the eye may lead to serious sequelae. Enlarged lesions can arise in humans suffering from immunosuppressive conditions, burns, or atopic dermatitis (Robinson and Lyttle, 1992; Lederman et al., 2007b). Also, lesion healing can be complicated by bullous pemphigoid (Murphy and Ralfs, 1996). Reinfections have been documented (Becker, 1940).

Human pseudocowpox lesions usually appear on the hands and are relatively painless but may itch. The draining lymph node may be enlarged. The nodules are gradually adsorbed and disappear in 4 to 6 weeks (Carson and Kerr, 1967).

Human bovine papular stomatitis lesions occur on hands, diminish after 14 days, and are no longer evident 3 to 4 weeks after onset (Carson and Kerr, 1967).

Sealpox virus causes lesions clinically similar to orf and was initially identified in a marine mammal research technician (Clark et al., 2005).

Gross Lesion Pathology

The orf lesion characteristically goes through a maculopapular stage in which a red center is surrounded by a white ring of cells which is surrounded by a red halo of inflammation as shown in Fig. 10; however, patients usually present later when the lesion is at the granulomatous or papillomatous stage 3 to 4 weeks following the initial infection. It takes the lesion at least 4 to 7 weeks to heal (Robinson and Lyttle, 1992).

In human pseudocowpox, milker's nodules are first observed as round cherry-red papules; these develop into purple, smooth nodules of up to 2 cm in diameter and may be umbilicated. The lesions rarely ulcerate (Becker, 1940).

The lesions of human bovine papular stomatitis appear as circumscribed wart-like nodules which gradually enlarge until they are 3 to 8 mm in diameter (Carson and Kerr, 1967).

Diagnosis and Differential Diagnoses

Diagnosis of parapoxvirus infection is by clinical (lesion morphology) and epidemiological evidence (recent contact with cattle, sheep, or pinnipeds) and EM of negative-stained lesion material (presence of ovoid particles with crisscross spindles) (Groves et al., 1991). Across the species of this genus are probably multiple distinct genotypes. More recently, PCR assays have been developed to help diagnose parapoxvirus infections (Inoshima et al., 2000; Becher et al., 2002; Torfason and Gunadottir, 2002; Gallina et al., 2006; Nitsche et al., 2006a).

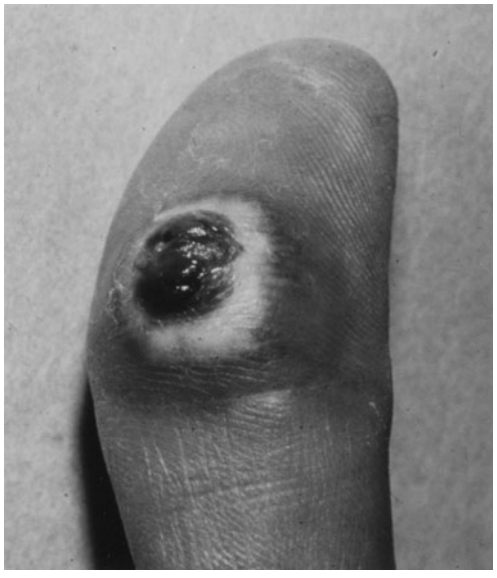

FIGURE 10 A typical orf lesion at the target stage of development. Courtesy of Andrew Mercer.

Without knowing the animal source of the infection, orf cannot be differentiated from milker's nodule based on clinical findings, histology, or EM (e.g., disease acquired from sheep or goat is orf and from cattle is milker's nodule or possibly bovine papular stomatitis) (Groves et al., 1991). PCR-based diagnostic assays are important for identification of the parapoxvirus which is causing the lesion.

Atypical giant orf lesions in patients who are immunocompromised or suffering from burns or atopic dermatitis may be confused with pyogenic granuloma (Tan et al., 1991; Robinson and Lyttle, 1992; Lederman et al., 2007b).

Yatapoxviruses

The genus *Yatapoxvirus* has two members, tanapoxvirus and Yaba monkey tumor virus, which are serologically related. Originally thought to be a third species of yatapoxvirus, Yaba-like disease in monkeys is caused by the same virus that causes tanapox in humans (Downie, 1972; Downie and Espana, 1972, 1973) as evidenced by DNA restriction endonuclease analysis of genomic DNA (Ropp et al., 1999) and more recently by genomic sequencing (Lee et al., 2001; Nazarian et al., 2007). Yaba monkey tumor virus has been isolated only from animal handlers, whereas tanapox virus has been found to be acquired by humans in riverine or forested areas of Africa (Downie, 1972; Downie and Espana, 1972; Dhar et al., 2004).

Pathogenesis

Transmission

Tanapox virus infection may occur via scratches or possibly via arthropod vectors. Yaba monkey tumor virus is a very rare infection of animal handlers at nonhuman primate facilities. There is no evidence for human-to-human transmission with either virus, and autoinoculation of virus to other areas of the body is not common.

Lesion Histopathology

Little is known concerning the pathology of yatapoxviruses except from the study of Yaba monkey tumor virus in nonhuman primate models (Niven et al., 1961).

Epidemiology

Geographic Distribution

Tanapox is endemic in equatorial Africa (Jezek et al., 1985). The animal reservoir is not known.

Yaba monkey tumor virus appeared in primate colonies, but has yet to be seen in nature.

Prevalence and Incidence

In the town of Lisa (population, 70,000) in northern Zaire, 264 laboratory-confirmed tanapox cases were observed between 1979 and 1983 (Jezek et al., 1985). More recently, a case in a traveler returning from an extended stay in a forested area of Republic of Congo was reported (Dhar et al., 2004).

There have been no reported human cases of Yaba monkey tumor virus in over 2 decades.

Diagnosis

Clinical Signs, Symptoms, and Severity

In most patients infected by the tanapox virus, fever (38 to 39°C) commenced 1 to 2 days prior to skin eruptions and was frequently accompanied by severe headache, backache, and prostration. In most patients, only a single lesion was observed which developed on parts of the body not usually covered by clothes. Multiple lesions, when observed, ranged from 2 to 10 in number. Regional lymph nodes became enlarged with lesion development. Lesions, nodular in nature, usually disappeared spontaneously within 6 weeks, unless there was a secondary infection (Jezek et al., 1985).

Gross Lesion Pathology

By the end of the first week after infection with tanapox virus, the lesion is greater than 10 mm in diameter, with a large erythematous areola several centimeters wide surrounded by edematous skin. The lesions can develop into large nodules but more likely ulcerate without pus. The maximum diameter of the lesion is reached in the second week (Jezek et al., 1985).

Diagnosis and Differential Diagnoses

Diagnosis of tanapox has historically been made by a combination of clinical (lesion character and number), epidemiologic (equatorial Africa), and laboratory (enveloped brick-shaped virions in lesion material) findings (Jezek et al., 1985). Patients with multiple lesions can be misdiagnosed as having monkeypox (see above). Today, PCR analysis of lesion material can be very useful in providing speciation of the poxvirus causing the infection (Dhar et al., 2004; Zimmermann et al., 2005).

PREVENTION AND THERAPY

Except for molluscum contagiosum, most current poxvirus infections of humans are zoonoses which fail to maintain human-to-human transmissions. Also, since molluscum contagiosum virus causes a benign, self-limiting disease in immunocompetent patients, there is a perceived lack of urgency for development of prevention strategies. A notable exception among the zoonotic poxviruses is monkeypox, which is capable of human-to-human transmission and causes significant human disease. Due to concerns over smallpox (vaccinia) vaccine-related adverse events in immunocompromised persons and persons in other high-risk groups (e.g., pregnancy, eczema), vaccination with currently available, licensed smallpox vaccines has not been employed widely as a strategy to help prevent monkeypox infection in Africa. These facts support the need for development of safer vaccines and effective antiorthopoxviral therapies.

The management of orthopoxvirus, parapoxvirus, and yatapoxvirus infections is currently largely supportive. There are no systemic or topical chemotherapeutic agents commercially available, FDA approved, and licensed to treat poxvirus infections. Vaccinia immune gamma globulin (VIG) has been useful in a number of human vaccinia virus infections (Goldstein et al., 1975; Bray, 2003; Pepose et al., 2003; Hopkins and Lane, 2004; Wittek, 2006) and is licensed for use in treatment of vaccine-related adverse events associated with direct viral replication. Prevention of secondary bacterial infections through the use of antibiotic ointments is also an option. In the case of cowpox, steroids are contraindicated and may exacerbate the illness (Baxby et al., 1994). Current ongoing research has identified several potential antiviral therapies for treatment of serious orthopoxviral infection, some of which are in clinical trials (ST-246 and cidofovir) (Toutous-Trellu et al., 2004; Bailey et al., 2007). Successful treatment of a recent case of eczema vaccinatum in a young boy included investigational use of intravenous VIG, cidofovir, and ST-246 (Vora et al., 2008).

Because of the chronic nature of molluscum contagiosum, curettage and cryotherapy of lesions are therapeutic options.

Cryotherapy is relatively painless and cost-effective and yields good cosmetic results; with patients infected with HIV, this treatment approach has the added advantage of mitigating the risk of disease transmission to medical personnel. Removal of giant molluscum lesions, but not regular lesions, usually results in scar formation. More-recent studies have suggested that other topical treatments (cidofovir and imiquimod) may be effective therapeutics for molluscum contagiosum and orf virus infections, even with immunocompromised patients (Geerinck et al., 2001; Berman, 2002; De Clercq, 2002; Trizna, 2002; Garland, 2003; Bikowski, 2004; Arican, 2006; Lederman et al., 2007b).

WILL SMALLPOX OR A SMALLPOX-LIKE DISEASE APPEAR IN THE FUTURE?

With the certification of global eradication of smallpox, the obvious need for preventive immunization with the smallpox (vaccinia) vaccine ceased, and a growing percentage of the world's population now lacks protective immunity to smallpox and related orthopoxviruses. Thus, the world's population is more vulnerable to possible reintroduction of variola virus or the evolution of related orthopoxviruses that might maintain human-to-human transmission. The risk of reintroduction of variola virus is thought to be low. Persistence of infectious variola virus in the soils or waters of temperate or tropical climates is considered unlikely, leaving only the remote possibility of preservation of virus in corpses locked in permafrost (Fenner et al., 1988). Examination of frozen smallpox patient corpses failed to yield evidence of infectious virus (Enserink and Stone, 2002). A second possible source of variola virus is from accidental laboratory infections. This possibility is also considered remote, as over the last 5 decades, the number of laboratories carrying out variola-virus related research has been reduced to the two WHO-sanctioned collaborating centers, and the biosafety features of these facilities are substantial. Reintroduction of variola virus into the human population from hypothetical clandestine stocks of variola virus held by groups that have not complied with WHO recommendations is a possible concern. Ultimately, the hypothetical reintroduction of variola virus into the world could be envisioned to have the greatest impact on populations of persons with the least access to medical infrastructure, including access to vaccines. Under the current political climate, those populations with the least potential to respond to a smallpox pandemic would not be expected to be the intended target for anticipated terrorism.

Perhaps a more likely scenario is the evolution of a poxvirus that efficiently infects and transmits in human populations with associated severe morbidity and mortality. Based on our knowledge of poxvirus biology, the *Orthopoxvirus monkeypox* would be a potential candidate for adaptation for efficient human-to-human transmission. Currently monkeypox virus infects humans causing a disease that clinically is almost indistinguishable from smallpox but without the same level of mortality and transmissibility. Although poxviruses have a relatively low mutation frequency compared to RNA viruses, the availability of a growing human population lacking specific antiorthopoxvirus immunity and the growing population of persons with impaired immune responsiveness is providing the opportunity for more frequent monkeypox virus infections of humans in central Africa, which possibly could allow for the selection of a virus more virulent and more transmissible to humans.

A novel formulation of smallpox (vaccinia virus) vaccine (Acambis 2000) has been stockpiled by the United States in the event mass vaccinations are required to contain an orthopoxvirus pandemic. However, the presence of persons with contraindications for smallpox vaccination (e.g., organ transplantation, HIV positivity, atopic dermatitis, pregnancy, etc.) makes rapid, large-scale immunizations with a live virus vaccine problematic. As there is currently no FDA-licensed antiorthopoxviral therapeutic, the only treatment for progressive vaccinia or eczema vaccinatum is VIG, which is not always efficacious.

Due to concerns for the immunocompromised population and persons with other risk factors for smallpox vaccination, there is much ongoing research into the development of third-generation vaccines which would use highly attenuated strains of vaccinia such as modified vaccinia virus Ankara (MVA). The replication-defective MVA strain was identified after serial passages (572 times) of vaccinia on chicken embryo fibroblasts (Mayr et al., 1975; Mayr et al., 1978). MVA was first used as a vaccine at the end of the smallpox eradication campaign in Germany (Stickl et al., 1974). Several studies have shown the safety of MVA in immunocompromised animal models and more recently demonstrated that MVA vaccination provided protective immunity to monkeypox virus challenge in nonhuman primate models (Stittelaar et al., 2001; Stittelaar et al., 2005; Edghill-Smith et al., 2003; Earl et al., 2004; Earl et al., 2007). Although a good animal model for variola virus infection does not exist, current research utilizing in vitro methods determined that MVA vaccination of humans induces an immune response capable of neutralizing variola virus as efficiently as vaccination with replication-competent vaccinia (I. K. Damon et al., unpublished data). Another possible vaccine candidate is the replication-competent attenuated vaccinia strain LC16m8, developed in Japan by passaging (45 times) vaccinia Lister through rabbit kidney cells (Kenner et al., 2006). LC16m8 was selected for its decreased neurovirulence and was safely used to vaccinate over 50,000 children during the 1970s. In recent years, Japan has increased its national stockpile of LC16m8 and more-detailed animal and clinical studies have been initiated (Morikawa et al., 2005; Empig et al., 2006; Kenner et al., 2006; Saijo et al., 2006). Other future candidate vaccines include NYVAC and dVV-L, attenuated vaccinia strains derived from specific deletions of genes (Tartaglia et al., 1992; Paoletti et al., 1995; Coulibaly et al., 2005). Research continues to identify an efficacious orthopoxvirus vaccine that is safe for use by immune-impaired persons.

CONCLUSIONS

Eleven poxviruses have been documented to infect humans (Table 3). These poxvirus diseases are primarily zoonoses, with the exception of molluscum contagiosum and the eradicated variola virus. Typically, the zoonotic poxviruses fail to maintain human-to-human transmission. When human-to-human transmission has been recognized, as in the case of monkeypox, transmission has not been documented to continue for more than 6 generations. Most examples of human infections with various poxviruses occur through minor abrasions in the skin. The parapoxvirus orf and molluscum contagiosum virus cause the most frequent poxvirus infections worldwide; however, the incidence of monkeypox in sub-Saharan Africa is imperfectly understood and may be on the rise. Unless an individual is immunocompromised, the zoonotic poxvirus infections usually resolve themselves uneventfully; however, the orthopoxvirus monkeypox is again an exception, with mortality rates as high as ~12%. Smallpox (vaccinia) vaccination successfully controlled outbreaks of

the orthopoxvirus variola, allowing for its eradication, and could probably be used to help prevent infection with multiple related orthopoxviruses. However, vaccination utilizing existing licensed smallpox vaccine would be problematic as a means of control of human monkeypox in Africa due to the growing population with contraindications for smallpox vaccination. Current strategies for preventing human poxvirus infections are ones that stress awareness of the potential for infection and possible behavioral modifications to reduce risk of infection. As might be expected, specific treatment modalities are focused mainly on molluscum contagiosum, which is relatively common among children, adults, and severely immunocompromised HIV-positive patients. Current studies are focused on third-generation vaccines against orthopoxviruses (e.g., monkeypox virus), which promise fewer risks of adverse vaccination events, and development of efficacious antiviral therapeutics.

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Parvoviruses

STANLEY J. NAIDES

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The family *Parvoviridae* is composed of small, nonenveloped, linear single-stranded DNA viruses. Parvo derives from the Latin "parvus," meaning small. The family is composed of two subfamilies based on host range: members of the *Parvovirinae* infect vertebrates and members of the *Densovirinae* infect invertebrates. The subfamily *Parvovirinae* comprises five genera: *Parvovirus*, *Dependovirus*, *Erythrovirus*, *Amdovirus*, and *Bocavirus*. The genus *Parvovirus* consists of those members of the family that infect nonerythroid mammalian host cells and replicate autonomously. Dependoviruses require the presence of a helper virus such as adenovirus or herpesvirus to replicate. *Erythrovirus* consists of those members of the family that infect erythroid mammalian host cells and can replicate without a helper virus, i.e., they are autonomous. Previously, the prototype erythrovirus, B19, was classified with the parvoviruses, but the erythroviruses have been separated in recent years in recognition of their tropism for erythroid precursors and the ability of B19 to cause erythematous rashes, hence erythroviruses. The genus *Amdovirus* consists of the Aleutian mink disease virus and related strains. *Bocavirus* consists of bovine parvovirus type 1, canine minute virus, and a recently reported human bocavirus. The subfamily *Densovirinae* is composed of those autonomously replicating invertebrate viruses previously known as densonucleosis viruses because of characteristic nuclear changes in the host cell occurring during infection.

In 1975, Yvonne Cossart and colleagues reported the discovery of parvovirus-like particles in human serum originally screened for hepatitis B surface antigen (HBsAg). In a screen of 3,219 sera received in a routine clinical laboratory, three sera were found to be positive for HBsAg by electrophoresis, but not by more sensitive reverse passive hemagglutination tests or radioimmunoassays (RIA) (Vandervelde et al., 1974). Two of the three sera were positive for HBsAg by electron microscopy (EM). One of the three was included in a control panel of sera for HBsAg tests. This serum was labeled no. 19 in panel B. Cossart and her colleagues realized that the sera that were positive in electrophoresis assays for HBsAg but negative in the more sensitive tests contained a new viral antigen. The antibody source for the detection of HBsAg by the electrophoresis tests was human serum which contained antibodies against HBsAg as well as antibodies against the new antigen. The antibody source used for detection of HBsAg in the reverse passive hemagglutination tests and RIA was hyperimmune antiserum raised in animals using a purified preparation of HBsAg. Thus, these animal antisera did not contain antibodies against the new antigen. The lack of identity of the new antigen to HBsAg was demonstrated in Ouchterlony gel diffusion tests (Fig. 1) (Cossart et al., 1975). EM of the serum containing the new antigen revealed spherical particles and empty shells typical of the *Parvoviridae*. On cesium chloride gradients, the antigen banded at a buoyant density of 1.36 to 1.40, also typical of *Parvoviridae*. A human parvovirus had not been described previously. In deference to the serum identifier of the initial positive serum, the new virus was named B19. The antigen was next identified in sera from nine healthy blood donors, one patient with acute hepatitis, and one recent renal graft transplant recipient. Convalescent-phase sera from four of these individuals demonstrated loss of virus with concomitant seroconversion. While Cossart et al. were unable to associate the presence of virus with a specific illness, initial studies found 30% of adults had antibodies against this virus. Cossart et al. noted the similarity of their new antigen to particles in feces described in patients with acute gastroenteritis (Paver et al., 1973; Pattison, 1988). This chapter will focus on the two known human parvoviruses: B19 and human bocavirus.

CHARACTERISTICS OF PARVOVIRUS B19

Parvovirus B19 is a nonenveloped, icosahedral virus that may appear spherical on EM. The particle measures approximately 23 nm in diameter, although a range of particles from 20.5 to 25 nm have been described. The parvovirus B19 genome is a 5.6-kb, single-stranded DNA molecule characterized by imperfect palindromes at both the 3′ and 5′ ends. The palindromic ends form terminal hairpin loops (Summers et al., 1983; Cotmore and Tattersall, 1984; Astell and Blundell, 1989). While most autonomous parvoviruses possess unique sequences at either terminus, B19 differs in that its termini are inverted terminal repeats (Deiss et al., 1990). In this respect, B19 resembles adeno-associated virus of the sister genus *Dependovirus* (Berns and Hauswirth, 1984). As a result of this structure, B19 is able to package either a plus- or a minus-sense DNA strand in a given virion, yielding progeny viral stocks with approximately equal numbers of plus- and minus-sense genomes. A single copy of the genome is encapsidated.

B19 replication follows a modified rolling hairpin model of replication characteristic of the autonomous parvoviruses (Tattersall and Ward, 1976). The imperfect palindrome at

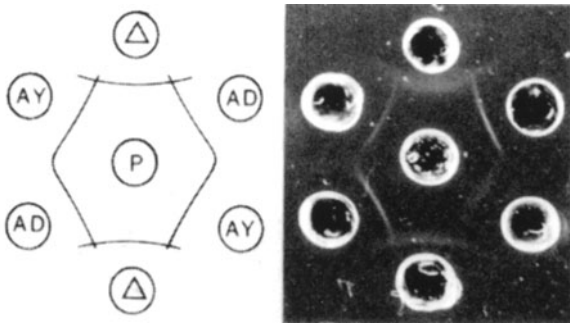

FIGURE 1 Ouchterlony gel diffusion assay showing newly discovered B19 antigen (Δ) nonidentity with HB antigen subtypes ad and ay, using anti-B19 antibody positive antiserum P (reprinted from Cossart et al., 1975, with permission from Elsevier).

the 3′ end of the molecule forms a region of double-stranded DNA that primes 3′ elongation during replication. As the 3′ complementary strand (C_1) elongates, it unfolds the 5′ end of the parental strand (V_1). This represents the monomeric replicative form. The 3′ end of C_1, which is now complementary to the parental 5′ V_1 hairpin, is able to fold back on itself and continue elongation. As C_1 elongates, a complementary strand (V_2) with the same sequence as the parental virus (V_1) is made. As the 3′ end elongation continues, a second complementary strand (C_2) is synthesized. The resulting form is the dimeric replicative form. The viral nonstructural protein NS1 serves as the "nickase," which reduces the replicative forms to progeny virus. Nickase reduction results in two distinct configurations of the distal 375-nucleotide palindromes, which are inverted complements of each other. These alternate configurations of the terminal hairpins have been found in all parvoviruses analyzed so far and are referred to as "flip" and "flop" (Astell, 1990; Carter et al., 1990). While parvovirus B19 is autonomous in that it does not require a helper virus, productive replication is still restricted to rapidly dividing cells, where cellular factors required for viral replication are found in the nuclei. Parvovirus B19, like other parvoviruses, replicates and assembles in the cell nucleus. Upon histological analysis, a parvovirus infection may be suspected on the basis of intranuclear inclusions characterized by a peripheral nuclear presence.

The virus has been sequenced, and while sequence variation between isolates has been identified, there is no association with clinical manifestations (Shade et al., 1986; Blundell et al., 1987; Erdman et al., 1996; Johansen et al., 1998; Takahashi et al., 1999). The virus employs a somewhat simple coding strategy. Interposed between the palindromes, a single strong promoter at map unit 6 initiates transcription of the left-hand nonstructural protein gene region and the right-hand structural gene region (Blundell et al., 1987). The nonstructural protein NS1 is approximately 74 kDa and is encoded between nucleotides 435 and 2448. NS1, as demonstrated for homologous nonstructural proteins in other parvoviruses, has a DNA-binding domain, an ATP-binding site, and helicase activity, thereby providing the nickase activity necessary to reduce replicative forms to progeny virus (Willwand et al., 1997; Corbau et al.,1999; Dettwiler et al., 1999). NS1 may also participate in the assembly of viral DNA into mature viral capsids. Cotmore and Tattersall (1989) identified NS1 on the external surface of mature B19 viral particles covalently linked to virion DNA.

Structural proteins VP1 (84 kDa) and VP2 (58 kDa) are encoded by nucleotides 2444 to 4786 and 3125 to 4786, respectively (Cotmore et al., 1986; Ozawa and Young, 1987). Both structural proteins are encoded in the same open reading frame, but VP2 results from an alternatively spliced transcript. mRNA for VP2 is initiated at an ATG codon at nucleotides 3125 to 3127 (Ozawa et al., 1987a). Digestion of B19 isolates with panels of restriction endonucleases has revealed strain variation and sequence drift, but the restriction digest variation was limited and not associated with consistent strain patterns, serological variation, or differences in clinical syndromes (Mori et al., 1987; Erdman et al., 1996; Johansen et al., 1998; Takahashi et al., 1999). However, subsequent detailed DNA sequence analysis of B19 isolates identified strains with 10 to 12% sequence variation and clustering into three separate genotypes (Servant et al., 2002). The original B19 isolates comprise genotype 1, which is found worldwide. Genotype 2 consists of A6, LaLi/K71, and Vx strains (Nguyen et al., 2002, Hokynar et al., 2002). Genotype 3 consists of the V9 virus and related isolates (Nguyen et al., 1999). Protein homology between genotypes is 96 to 97%, and no antigenic differences have been identified (Heegaard et al., 2002). Similarly, no differences between biological behavior or medical presentation have been identified to date.

PATHOGENESIS OF PARVOVIRUS B19 INFECTION

Much of the early knowledge of the natural history of B19 infection was based upon studies of experimental infection in healthy volunteers conducted by Mary Anderson and her colleagues at the Common Cold Research Unit, Harvard Hospital, in Salisbury, England (Anderson et al., 1985a). Plasma containing B19 was obtained from a healthy blood donor. The presence of other infectious agents was ruled out by inoculation of samples of this plasma into an array of bacterial and viral screening tests. The plasma was diluted in Hanks' buffered saline containing 0.2% bovine serum albumin, and 0.5 ml of diluted plasma was inoculated into each nostril of a volunteer. Volunteers with preexistent anti-B19 antibodies did not develop viremia. Virus was first detected 6 days after inoculation in previously seronegative individuals. Peak titers of virus were reached 8 and 9 days postinoculation. Viremia was present for up to 7 days. Virus was detected in nasal washes and gargle specimens between days 7 and 11 postinoculation, during the same period as the viremia. Virus was not detected in urine or fecal specimens from any of the volunteers, except for one woman whose urine was contaminated with menstrual blood. High-titer immunoglobulin M (IgM) antibody to B19 developed during the second week after inoculation. Anti-B19 IgG antibody began to develop at the end of the second week or early in the third week after inoculation. In one individual with a trace amount of preexisting anti-B19 IgG, increased levels of IgG antibody were detected at the time of a small IgM antibody response 9 to 10 days after inoculation. None of the volunteers with a significant anti-B19 IgG antibody level prior to inoculation developed a subsequent IgM antibody response.

The clinical illness associated with experimental B19 infection was biphasic (Anderson et al., 1985a). In some individuals, the viremia was asymptomatic, but in others, it was associated with a flu-like illness consisting of malaise, myalgia, and/or transient fever. Several individuals experienced headache, pruritus, and chills. The second phase of the illness began towards the end of the second week postinoculation and was characterized by rash, arthralgia, and arthritis.

Hematological alterations associated with B19 infection were noted by Anderson and her colleagues. There was an absolute areticulocytosis from the period of peak viremia until several days after the onset of the antibody response. The hemoglobin fell during the week after onset of areticulocytosis, but the decrease was not clinically significant. Neutropenia was detected as early as day 8 postinoculation and persisted through the second week following infection. Lymphocyte and platelet counts were transiently depressed during the second week postinoculation. No significant trends were observed in the monocyte, basophil, or eosinophil counts. The incubation period between inoculation and onset of symptoms in natural infections can be inferred to range from 6 to 18 days based upon the volunteer study and epidemiological studies of B19 outbreaks; however, studies of some outbreaks have suggested that the incubation period may be as long as 28 days. Individuals are infectious during the period of viremia. However, many patients will not present until the onset of either rash or joint symptoms, at which point they are no longer infectious. The onset of rash, polyarthralgia, or polyarthritis is temporally associated with the development of an anti-B19 antibody response that results in clearance of viremia and cessation of viral shedding (Joseph, 1986). Anti-B19 IgM antibody may be present for up to 2 months but declines thereafter. The anti-B19 IgG antibody response is long-lived, and seroprevalence in the adult population ranges from 40 to 60%. Acquisition of IgG antibody to B19, suggesting infection, is accelerated after 5 years of age when many children first enter school (Anderson et al., 1986).

CLINICAL MANIFESTATIONS OF PARVOVIRUS B19 INFECTION

Transient Aplastic Crisis

After Cossart's report, parvovirus B19 was a virus in search of a disease. While screening serum in a clinical laboratory for evidence of parvovirus B19 infection, Pattison and his colleagues observed six patients with sickle cell disease in aplastic crisis who had evidence of a recent B19 infection (Pattison et al., 1981). Aplastic crisis in sickle cell disease was first described in 1950 as an acute fall in hemoglobin associated with cessation of new erythrocyte formation, or areticulocytosis. Following areticulocytosis lasting approximately 7 to 10 days, a brisk reticulocyte rebound occurred with eventual return of hemoglobin to baseline levels. While the etiology of aplastic crisis in sickle cell anemia and other hemolytic anemias was not known, an infectious agent was suspected because the crisis usually occurred only once in any given individual and occasionally occurred in outbreaks within chronic hemolytic anemia clinic patient populations. Shortly after Pattison's observation, Serjeant and his colleagues confirmed that B19 was the cause of an epidemic of aplastic crisis in Jamaica between 1979 and 1981 (Serjeant et al., 1981). Subsequent studies demonstrated that B19 infection may cause aplastic crisis in chronic hemolytic anemia regardless of the anemia's underlying etiology. In short order, B19-induced aplastic crisis also was found to occur in individuals with hereditary spherocytosis, alpha and beta thalassemias, pyruvate kinase deficiency, glucose-6-phosphate dehydrogenase deficiency, pyrimidine 5′-nucleotidase deficiency, hereditary stomatocytosis, autoimmune hemolytic anemia, and hereditary erythrocytic multinuclearity associated with a positive acidified serum (Ham's) test (Pattison et al., 1981; Duncan et al., 1983; Rao et al., 1983; Davidson et al., 1984; Evans et al., 1984; Green et al., 1984; Kelleher et al., 1984; Bertrand et al., 1985; Summerfield and Wyatt, 1985; Tsukada et al., 1985; Lefrere et al., 1986a, 1986c; Lefrere et al., 1986b; Lefrere et al., 1986d; Lefrere et al., 1986f; Saarinen et al., 1986; Takahashi et al., 1986; West et al., 1986; Hanada et al., 1988; Rappaport et al., 1989; Rechavi et al., 1989; Goldman et al., 1990; Mabin and Chowdhury, 1990). All of these patients shared the need for brisk reticulocytosis to maintain their hemoglobin levels in the face of shortened erythrocyte survival. During the period of areticulocytosis, individuals usually required transfusion support. Examination of the bone marrow revealed growth arrest at the giant pronomoblast stage of erythrocyte development. Marginal intranuclear inclusions may be seen and represent accumulated B19 virus (Burton, 1986; Caul et al., 1988; Knisely et al., 1988).

Erythema Infectiosum

Since the end of the 19th century when pediatricians enumerated the rash illnesses of childhood, the fifth rash—erythema infectiosum or fifth disease—has been a well-described clinical entity. In May 1983, an outbreak of a rash illness in a primary school in North London was described that had typical features of erythema infectiosum and was confirmed to be caused by B19 infection (Anderson et al., 1984). The majority of the children had the typical rash of classic erythema infectiosum characterized by bright red "slapped cheeks." The rash may also be seen on the torso and extremities (Color Plate 8). The exanthem is frequently lacy or reticular in pattern but may be macular, maculopapular, or occasionally, vesicular or hemorrhagic. It is pruritic in approximately half the cases. In the majority of children, the rash recurs after initial resolution; recurrence is usually precipitated by sun exposure, hot baths, or physical activity. Rash recrudescence may occur for weeks or months following the acute infection, but children are not infectious during episodes of recrudescence. Infection in children may be asymptomatic, and when symptoms do occur, they tend to be mild. These include sore throat, headache, fever, cough, anorexia, vomiting, diarrhea, and arthralgia (Brandrup and Larsen, 1976; Cramp and Armstrong, 1976; Lauer et al., 1976; Shneerson et al., 1980; Anderson et al., 1983; Andrews et al., 1984; Clarke, 1984; Okabe et al., 1984; Mynott, 1985; Nunoue et al., 1985; Plummer et al., 1985; Shiraishi et al., 1985; Chorba et al., 1986; van Elsacker-Niele and Anderson, 1987; Mansfield, 1988; Naides et al., 1988b). At the time of presentation, children with a rash usually have anti-B19 IgM antibodies (Shiraishi et al., 1985).

Parvovirus Arthritis

Erythema infectiosum may also be seen in adults not previously infected. In adults, the rash tends to be subtler, and the bright red "slapped cheeks" are absent. When adults are symptomatic, they tend to have a more severe flu-like illness in which polyarthralgia and joint swelling are more prominent. For example, Ager and colleagues observed adult involvement in an outbreak of erythema infectiosum in Port Angeles, Washington, in 1961 to 1962. Subjects were identified by the presence of a typical rash. Only 5.1% of children under 10 years of age had joint pain, and 2.8% had joint swelling. In children infected during their adolescent years, joint pain and swelling occurred in 11.5% and 5.3%, respectively. However, in the 20-years-old or older age group, joint pain occurred in 77.2% and joint swelling in 59.6% (Ager et al., 1966).

White and his colleagues demonstrated that B19 could cause a chronic rheumatoid-like arthropathy (White et al.,

1985). Sera were collected from individuals presenting at an "early synovitis" clinic at the Royal National Hospital for the Rheumatic Diseases, in Bath, England, beginning in mid-1979. Primary care physicians were invited to refer patients to the clinic as soon as possible after the onset of acute joint symptoms. Traditionally, referral would wait until it became clear that joint symptoms had become chronic, usually a period of 3 months at the time of the study. Current criteria for rheumatoid arthritis classification established by the American College of Rheumatology for study subjects with polyarthritis has been shortened in duration to 6 weeks of joint symptoms, making inclusion of B19 arthritis even easier (Arnett et al., 1988; Silman, 1988; Naides et al., 1990). Sera from 153 patients with early synovitis were retrospectively tested when assays for parvovirus B19 became available. Nineteen patients had evidence of a recent B19 infection. Although 49 of the cohort were men, all 19 patients with evidence of a recent B19 infection were women. Eighteen of the infected patients presented with acute, moderately severe symmetric polyarthritis that usually started in the hands or knees, and within 24 to 48 h involved wrists, ankles, feet, elbows, and shoulders. The cervical spine was involved in two cases and the lumbosacral spine in one case. In three cases, the upper limbs alone were affected. All patients complained of joint pain, stiffness, and variable swelling. The acute polyarthropathy usually improved within 2 weeks but completely resolved in only two cases. In 17 cases, symptoms persisted for more than 2 months, and in three cases, they persisted for more than 4 years. Thirteen of the 19 B19-infected patients reported an influenza-like illness with malaise, fever, gastrointestinal symptoms, and/or rash. Two-thirds of the patients had episodic flares but remained symptomatic between flares. One-third had episodic flares but were symptom-free between flares. There was no associated long-term joint damage (White et al., 1985). The distribution of joint involvement and its symmetry may suggest a diagnosis of a rheumatoid arthritis (Reid et al., 1985; White et al., 1985; Woolf et al., 1989; Naides et al., 1990). Many patients experience morning stiffness. About half of the patients with chronic B19 arthropathy meet the criteria of the American Rheumatism Association for a diagnosis of rheumatoid arthritis (Arnett et al., 1988; Silman, 1988; Naides et al., 1990). Patients may have a transient expression of autoantibodies during acute infection, including rheumatoid factor, anti-DNA antibodies, and antilymphocyte antibodies (Luzzi et al., 1985, Semble et al., 1987; Naides and Field, 1988; Sasaki et al., 1989; Soloninka et al., 1989). While initial reports suggested that chronic B19 arthropathy was associated with the major histocompatibility antigen HLA DR4, as is seen in classic erosive rheumatoid arthritis, subsequent studies have demonstrated no increased association with DR4 (Klouda et al., 1986; Woolf et al., 1987). In apparently immune-competent individuals with chronic B19 arthropathy, B19 DNA has been found in bone marrow aspirates and synovium (Naides et al., 1991a; Foto et al., 1993). B19 DNA also has been detected using sensitive nested PCR techniques in synovium from healthy military recruits undergoing arthroscopy for trauma, suggesting that B19 may persist in a latent state (Soderlund, et al., 1997). While B19 may present with a pattern similar to that of rheumatoid arthritis, it has generally not been thought to cause classic, rheumatoid factor-positive rheumatoid arthritis (Naides, 1998). A report suggesting that B19 upregulates production of tumor necrosis factor alpha and interleukin 6—two cytokines central to inflammation in the joint in rheumatoid arthritis—remains unconfirmed (Takahashi et al., 1998).

Hydrops Fetalis

During a regional outbreak of erythema infectiosum in Scotland, six pregnant women were found to have serologically documented B19 infection. Four gave birth to healthy term infants, and two aborted grossly hydropic fetuses with anemia during the second trimester. There was evidence of fetal leukoerythroblastic reaction with eosinophilic changes in hematopoietic cell nuclei, hepatitis, and excess iron pigment in the liver. Hybridization with radiolabeled B19 DNA probes demonstrated B19 DNA in fetal liver, heart, thymus, kidney, adrenal, and placental tissues (Anand et al., 1987). Additional observations have confirmed the association of maternal B19 infection with fetal hydrops (Brown et al., 1984; Knott et al., 1984; Mortimer et al., 1985a; Bond et al., 1986; Gray et al., 1986; Anand et al., 1987; Carrington et al., 1987; Clewley et al., 1987; Matsunaga et al., 1987; Woernle et al., 1987; Anderson and Hurwitz, 1988; van Elsacker-Niele et al., 1989; Knisely, 1990; Salimans, 1990).

The B19-infected fetus is similar to those with chronic hemolytic anemia, in that red cell survival is only 45 to 70 days and fetal red cell mass increases 34-fold during the second trimester (Gray et al., 1987). The infected fetus develops B19-induced aplastic crisis, resulting in high-output cardiac failure with hydrops evidenced as soft tissue edema, ascites, pleural effusions, and in some cases, polyhydramnios on ultrasound examination. A rise in maternal serum alpha-fetoprotein during the first trimester and early second trimester may herald ultrasound evidence of fetal hydrops (Carrington et al., 1987). Fetal B19 infection was thought to be uniformly fatal until Woernle and colleagues reported in 1987 on four pregnant women with anti-B19 IgM-positive serology, one of whom delivered a stillborn hydropic fetus whose tissues were positive for B19 DNA by nucleic acid hybridization, while the other three IgM-positive women gave birth to healthy offspring. One of the neonates had anti-B19 IgM antibody-positive cord serum. A second apparently healthy neonate was anti-B19 IgM negative but IgG positive; the anti-B19 IgG antibody persisted in the infant's serum at 9 months of age, confirming that it was of fetal origin consistent with an in utero infection (Woernle et al., 1987). While microphthalmia with abnormal lens development has been reported in an abortus, congenital anomalies have not been a common feature of B19 infection (Weiland et al., 1987; Kinney et al., 1988). However, case reports suggest that occasional abortuses may show evidence of developmental anomalies and survivors of B19 infection in utero may demonstrate evidence of a congenital syndrome characterized by anemia, thrombocytopenia, and cardiac and hepatic dysfunction (Naides et al., 1988a). Viral cardiomyopathy has been reported as a mechanism of fetal hydrops (Naides and Weiner, 1989). Hepatic dysfunction has been described in neonatal as well as adult B19 infection (Naides, 1987; Naides et al., 1988a; Metzman et al., 1989). Whether developmental anomalies represent a direct viral effect or the indirect effect of severe illness during gestation remains to be determined. Fetuses have been successfully treated for anemia by in utero transfusion, with excellent salvage and outcome (Peters and Nicolaides, 1990; Soothill, 1990; Sahakian et al., 1991).

Bone Marrow Suppression

Individuals with congenital or acquired immune deficiency may fail to clear B19 viremia. These include patients with Nezelof's syndrome, hyper-IgM syndrome with CD154 (CD40 ligand) mutation, prior chemotherapy for lymphoproliferative disorders, immunosuppressive therapy for transplantation,

or AIDS (Kurtzman et al., 1987; Kurtzman et al., 1988; Kurtzman et al., 1989b; Graeve et al., 1989; Young et al., 1989; Frickhofen and Young, 1989, 1990; Chrystie et al., 1990; de Mayolo and Temple, 1990; Frickhofen et al., 1990; Rao et al., 1990; Naides et al., 1993; Blaeser et al., 2005). In the immunocompetent host, the IgM antibody response may last 2 months or more. Anti-B19 IgM antibody and acute-phase IgG antibody (less than 1 week postinoculation) recognize determinants on VP2. In convalescent-phase serum, anti-B19 IgG antibody recognizes determinants on the VP1 structural protein (Kurtzman et al., 1989a, 1989b). As B19 VP1 and VP2 are products of alternate transcription of the same open reading frame, VP1 contains an additional 227 N-terminal amino acids not present in VP2 (Shade et al., 1986). VP1 therefore contains unique determinants not present in the truncated form represented by VP2. These determinants may be in the unique nonoverlapping N-terminal region or, alternatively, represent conformational differences in the sequences shared between the two proteins. Western blot analysis of serum from individuals with congenital immune deficiency, prior chemotherapy, or AIDS demonstrated the absence of convalescent-phase anti-B19 IgG antibodies directed against VP1. These sera were unable to neutralize B19 virus in bone marrow in vitro culture systems despite having antibodies to VP2. While this work suggested that neutralizing determinants are unique to VP1, studies with synthetic peptides suggested that neutralizing determinants may also be found on VP2 (Kurtzman et al., 1989a; Sato et al., 1991a; Sato et al., 1991b).

Neutralizing activity to B19 is found in commercially available pooled immunoglobulin, since the seroprevalence of anti-B19 IgG antibodies in the adult population is 40 to 60% (Anderson et al., 1986; Frickhofen et al., 1990; Naides et al., 1993). Immunocompromised individuals may fail to mount a neutralizing antibody response to B19, allowing virus to persist in the bone marrow and cause chronic or intermittent suppression of one or more hematopoietic lineages. For example, B19 is a major cause of red cell aplasia in individuals with human immunodeficiency virus type 1 infection. Intravenous immunoglobulin may be employed to treat B19-associated bone marrow suppression and B19 persistence (Frickhofen et al., 1990). However, immunoglobulin therapy may not be universally successful in clearing B19 persistence, and retreatment may be necessary (Bowman et al., 1990). Intramuscular immunoglobulin therapy may be beneficial in the treatment of concurrent B19 infection in AIDS patients refusing intravenous immunoglobulin (Naides et al., 1993).

Less Common Presentations

A number of uncommon dermatological manifestations of B19 infection have been reported. A vesiculopustular eruption has been seen in adult patients with fifth disease. It is unusual because it combines the features of morbilliform and vesiculopustular lesions. Subepidermal edema, ballooning necrosis of the dermis, and a lymphohistiocytic infiltrate may be noted. The infiltrate may contain unusual binucleate giant cells. The vesicle itself may contain a neutrophilic infiltrate. Extravasation of erythrocytes into the dermis eventually gives the vesiculopustules a hemorrhagic appearance (Naides et al., 1988b). Purpura may be seen in the absence of thrombocytopenia (Lefrere et al., 1985; Mortimer et al., 1985b; Shiraishi et al., 1989). Some patients may have purpura as a result of thrombocytopenia (Kilbourne et al., 1987; Lefrere et al., 1989). The clinical appearance may suggest Henoch-Schönlein purpura (Lefrere et al., 1985, 1986e). A "gloves and socks" syndrome of acral erythema with sharp demarcation at the wrists and ankles, respectively, has been described, although this is not specific to B19 (Etienne and Harms, 1996; Stone and Murph, 1993; Drago et al., 1997).

B19 infection may present as isolated neutropenia, thrombocytopenia, or anemia or as idiopathic thrombocytopenic purpura (Anderson and Cohen, 1987; Van Elsacker-Niele et al., 1996). Finger and toe paresthesias, progressive arm weakness, encephalopathy, and aseptic meningitis have all been reported (Denning et al., 1987; Walsh et al. 1988; Faden et al., 1990; Faden et al., 1992; Suzuki, et al., 1995; Umene and Nunoue, 1995; Koduri and Naides, 1995). Carpal tunnel syndrome may occur as a result of wrist swelling (Samii et al., 1996). B19 infection may trigger fibromyalgia—a muscle pain and fatigue syndrome—but this appears to represent a nonspecific viral trigger (Berg et al., 1993). Self-limited benign acute lymphadenopathy, hemophagocytic syndrome, and hemophagocytic syndrome in association with lymphadenopathy resembling necrotizing lymphadenitis (Kikuchi's disease) have also been reported (Boruchoff et al., 1990; Muir et al., 1992; Tsuda et al., 1993; Watanabe et al., 1994; Shirono and Tsuda et al., 1995; Yufu et al., 1997).

Transiently abnormal liver enzymes have been observed in healthy adults with acute B19 arthropathy and in neonates surviving anemia and nonimmune hydrops secondary to B19 infection in utero. B19 infection has been associated with acute hepatitis and some cases of non-A, non-B, non-C acute fulminant liver failure with or without associated aplastic anemia (Naides, 1987; Tsuda 1993; Langnas et al., 1995; Yoto et al., 1996; Longo et al., 1998; Karetnyi et al., 1999). In tissues nonpermissive for virion production, alternative splicing of capsid transcripts prevents virion protein production (Brunstein et al., 2000). Production of NS1 in restricted infection induces apoptosis through mitochondrial stress pathways (Poole et al., 2004; Poole et al., 2006). There is a question as to whether B19 can precipitate cutaneous vasculitis, polyarteritis nodosa, or Wegener's granulomatosis (Corman and Dolson, 1992; Finkel et al. 1994; Nikkari et al., 1994; Nikkari et al., 1997; Leruez-Ville et al., 1994; Corman and Staud, 1995). A role for B19 infection in juvenile rheumatoid arthritis has been suggested. However, the arthritis is monoarticular or pauciarticular, in contrast to the symmetric polyarthritis seen in adults, suggesting that either the disease presents differently in children or that age is a confounding factor in that juvenile rheumatoid arthritis occurs at a time when children acquire B19 infection (Nocton, et al. 1993).

PREFERRED SITES FOR VIRUS ISOLATION

Virus is most easily isolated in high titers ($\geq 10^{11}$ particles/ml) from serum obtained from individuals with aplastic crisis or fifth disease, the latter prior to development of rash or arthritis. Virus may also be isolated from serum of individuals with persistent infection in an immunocompromised setting, but viral titers tend to be lower (10^5 to 10^6 particles/ml serum). Virus may be found in bone marrow aspirates during the period of areticulocytosis in individuals with aplastic crisis or in those with persistent virus infection. In immunodeficient individuals with persistent infection and in chronic B19 arthropathy, B19 may be detected in bone marrow even in the absence of detectable viremia (Rao et al., 1990; Foto et al., 1993). In fetal infection, virus has been isolated from cord serum, ascites, and amniotic fluid (Naides and Weiner, 1989). Small, round, parvovirus-like particles have been found

in stool from individuals with gastroenteritis, but B19 has not been isolated from such specimens nor has it been reported in stool from individuals with known B19 infection. Rather, the small, round, parvovirus-like particles found in stool may be a related human parvovirus with sequence homology similar to B19 (Oliver and Phillips, 1988; Turton et al., 1990). Detection of B19 DNA in urine has been reported in one case of neonatal B19 infection (Naides et al., 1988a).

VIRUS STABILITY

B19 is not affected by ether, chloroform, 0.25% sodium deoxycholate, RNase, micrococcal DNase, potassium iodide, or heating at 45°C for 30 min. It is readily inactivated when heated at 56°C for 5 min, by treatment with 1 mg of proteinase K/ml, or by treatment with 0.05 N NaOH. Prior treatment of B19 with either 0.05 N HCl or glycine-HCl, pH 2.8, is about 75% effective in inhibiting B19 growth in bone marrow culture (Young et al., 1984). B19 may be stored frozen at –85°C for indefinite periods without loss of virion infectivity. Special handling is not required, although repetitive freeze-thawing may result in some loss of virus titer.

B19 may survive the dry heat processing of factor VIII and IX concentrates at 80°C for 72 h (Lyon et al., 1989). Solvent-detergent inactivation of factor VIII concentrate with tri-(*n*-butyl) phosphate detergents will not inactivate B19, and terminal dry heating of these preparations at 100°C for 10 to 30 min or more has been recommended (Rubinstein and Rubinstein, 1990). Despite these efforts, B19 may be transmitted via pooled blood products (Saldanha and Minor, 1996; Erdman et al., 1997; Wu et al., 2005; Hattori et al., 2007; Parsyan and Candotti, 2007).

PROPAGATION OF B19 IN VITRO

Routine culture of parvovirus B19 in vitro is not available. All continuous cell lines tested failed to support B19 growth, including erythroleukemic cell lines such as K562 and HEL (Gallinella et al., 1995). B19 was first grown in primary bone marrow suspension cultures supplemented with erythropoietin (Ozawa et al., 1986). Optimally, the bone marrow should be derived from individuals with hemolytic anemia (e.g., sickle cell anemia) in whom the erythroid precursor pool is increased. Amplification of virus and the death of cells as a consequence of viral replication limit bone marrow culture as a source of virus production. The input-to-output ratio of virus is only approximately 1:50 for culture supernatants and 1:200 for total cultures (Ozawa et al., 1987b). Propagation of B19 in primary fetal liver cell culture and human cord blood culture has been reported but, as with bone marrow cultures, is not efficient (Brown et al., 1991; Srivastava et al., 1991). Two megakaryocytic leukemic cells lines, UT-7 and MB-2, will support long-term virus growth, but these systems are inefficient as well (Takahashi et al., 1989; Komatsu et al., 1991; Shimomura et al., 1992; Munshi et al., 1993). The difficulty in culturing B19 has led investigators to seek a readily renewable antigen source through recombinant DNA technology. An infectious B19 clone has been utilized in initial studies of the B19 life cycle (Zhi et al., 2004; Zhi et al., 2006).

PRESENCE OF B19 IN VARIOUS TISSUES

In the immunocompromised patient, B19 may be found continuously or intermittently in serum during periods of viremia. Virus may also be found in the bone marrow of these individuals during this period, presumably representing persistent infection. In fetal infections, virus may be found in serum, amniotic fluid, and fetal ascites, as well as an array of body tissues, including the liver, spleen, kidney, thymus, bone marrow, heart, and placenta. The period after maternal infection during which B19 can be recovered from fetal tissues is not entirely clear and may depend on the severity of infection, gestational age of the fetus at the time of infection, and whether transfusion therapy for the fetus is attempted. In apparently immune-competent individuals with chronic B19 arthropathy, B19 DNA has been found in bone marrow aspirates and synovium (Naides et al., 1991a; Foto et al., 1993). B19 DNA has been found in the bone marrow, synovium, and liver in individuals without apparent active clinical disease. Therefore, the presence of B19 DNA in a tissue sample should be diagnostically interpreted with caution. The presence of B19 DNA in tissues does not necessarily demonstrate active viral processes or disease causality.

LABORATORY DIAGNOSIS OF B19 INFECTION

A number of approaches have been used to diagnose parvovirus B19 infection.

EM

EM provides morphologic identification of the virus. Standard touch preparations that are negatively stained are made by allowing a liquid sample to dry on an EM grid that had been previously coated with a thin layer of plastic. After the sample has dried, it is stained with phosphotungstic acid or uranyl acetate, both of which are electron-dense material that accumulates around the particle to give a bas-relief appearance, or negative image. In the absence of endogenous antibody to B19, incubation of the sample with specific antiserum or monoclonal antibody to B19 may cause aggregation of viral particles which can be visualized by electron microscopic examination (Cohen, 1988).

A combined pseudoreplica-immunochemical staining technique offers the advantage of preserving the morphology while providing a specific serologic diagnosis (Naides and Weiner, 1989). This approach may be useful even in the presence of endogenous B19 antibodies that cause virus to aggregate, since antigenic sites for binding exogenous anti-B19 antibodies may still be available. Virus in various body fluids may be examined by this technique. A 25-µl sample is allowed to absorb into an agarose block, leaving the viral particles on the surface. The agarose is layered or coated with plastic, which is allowed to harden and then floated off the agarose, inverted, and applied to a support grid. The pseudoreplica may then be negatively stained with phosphotungstic acid or uranyl acetate. Immune EM may be performed on pseudoreplicated samples prior to negative staining. The samples for immune EM are applied to nickel support grids as above, then incubated ("blocked") with goat serum diluted 1:10 in 0.1 M phosphate-buffered saline with 0.1% glycine prior to incubation with anti-B19 monoclonal antibody. Samples are washed and then incubated with a second-stage antibody conjugated to colloidal gold to enable visualization of the antibody by EM. Samples are then negatively stained with uranyl acetate and carbon coated (Fig. 2) (Naides and Weiner, 1989). Observation of viral particles with specific colloidal gold-conjugated antibody allows species identification. B19 virus may be difficult to distinguish from enteroviruses on the basis of morphology

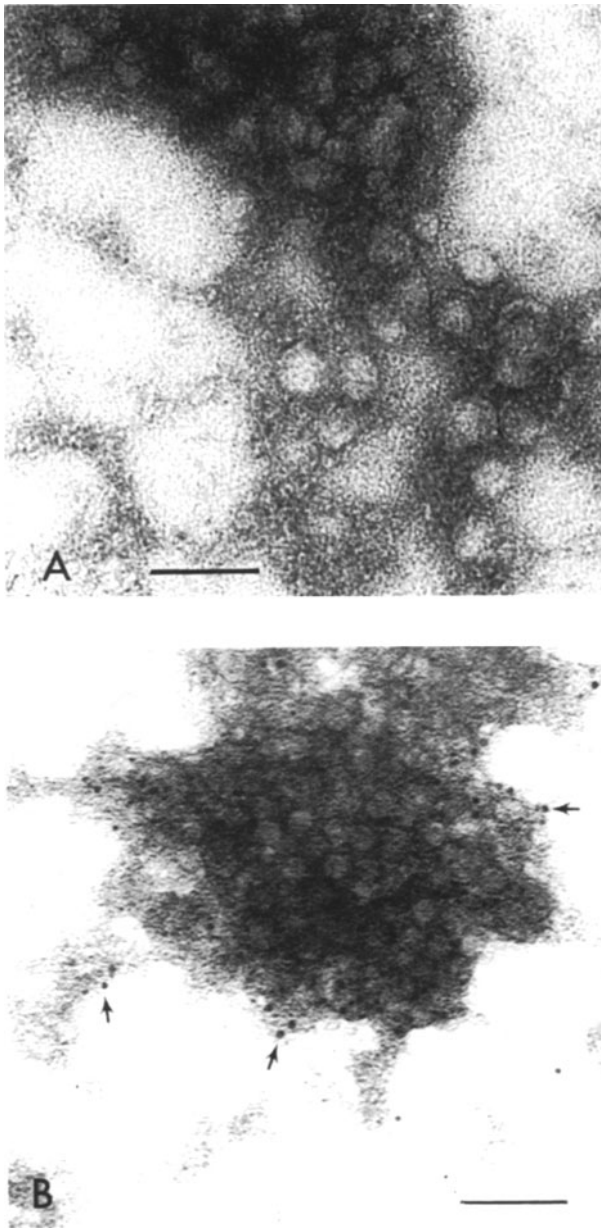

FIGURE 2 (A) Fetal ascites from a hydropic fetus showing viral particles without label, prepared by a pseudoreplica technique, and negatively stained by uranyl acetate. Original magnification, ×100,000; image magnification, ×280,000; bar, 50 nm. (B) The fetal ascites prepared by pseudoreplica technique and identified as B19 by indirect labeling with colloidal gold (arrow) before negative staining with uranyl acetate. Original magnification, ×50,000; image magnification, ×140,000; bar, 100 nm. (Reprinted with permission from Naides and Weiner, 1989.)

alone. B19 particles may exclude uranyl acetate or phosphotungstic acid, giving them a "full" appearance, or stain may enter the capsid, giving the appearance of an "empty" shell.

Immunoassay

Both RIA and enzyme-linked immunosorbent assays (ELISA) have been used to detect B19 antigen and specific antibody to B19. In antigen-capture assays, an anti-human IgM or IgG antibody is allowed to coat a solid phase. In the second step, a serum known to contain either anti-B19 IgM or IgG antibody is incubated on the plate, and excess antibody is removed by washing. In the third step, the test serum is added to allow capture of B19 antigen. Captured antigen is detected by a mouse monoclonal antibody to viral antigen followed by an anti-mouse antibody labeled with ^{125}I (RIA) or peroxidase (ELISA) (Anderson et al., 1986; Bell et al., 1989). Cohen and colleagues (1983) first described the antibody-capture assay for anti-B19 IgM antibody in which the serum to be tested for anti-B19 antibody is added in the second step, and a serum known to contain B19 virus is substituted in the third step. Detection of the captured antigen indicates the presence of antibodies to B19 virus in the patient's serum. Early reports of cross-reactivity between anti-B19 and anti-rubella antibodies were based on counter immunoelectrophoresis techniques, which have been replaced by RIA and ELISA methods. In the RIA and ELISA methods, cross-reactivity has not been a problem (Cohen and Shirley, 1985; Kurtz and Anderson, 1985; Cohen and Supran, 1987). Antigen capture followed by PCR amplification of captured viral DNA improves sensitivity (Karetnyi et al., 1999).

In response to the dependence on B19 viremic serum as a source of antigen, a number of recombinant antigens have been developed for B19 testing. B19 VP1 and VP2 proteins have been expressed in Chinese hamster ovary cells transfected with a B19 plasmid construct; the VP1 and VP2 self-assembled into empty capsids (Kajigaya et al., 1989). Transfected cell lysates were useful as an antigen source. VP1 and VP2 have been coexpressed in the baculovirus system as well (Brown et al., 1990), and B19 synthetic peptides, based upon sequence analysis of the viral capsid gene, have also been employed as an antigen source. Synthetic peptides provide approximately 90% sensitivity and specificity compared with serologic titers using native virus (Fridell et al., 1991). B19 antigen also has been prepared as fusion proteins expressed in *Escherichia coli* (Morinet et al., 1989). A kit using baculovirus-expressed empty capsids containing only B19 VP2 for serologies has been approved by the Food and Drug Administration for diagnostic testing in the United States. Recombinant empty capsids have been used as a source of antigen and are the basis of current commercially available tests (Bruu and Nordbo, 1995; Sloots and Devine, 1996; Schwarz et al., 1997; Jordan, 2000; Butchko and Jordan, 2004).

IgG1 is the predominant IgG subclass in B19 infection directed against VP1 and VP2. IgG3 to VP1 and VP2 is associated with acute infection. IgG4 specific to VP1 occurs months after infection (Franssila et al., 1996).

Nucleic Acid Probes

B19 DNA may be detected by hybridization with cDNA probes, riboprobes (synthetic RNA), or synthetic oligonucleotide probes. B19-specific probes have been used to identify virus by in situ hybridization (Hassam et al., 1990; Schwarz et al., 1991). Anderson and colleagues (1985b) and Clewley (1985) first reported detection of B19 viral DNA using molecularly cloned probes that were ^{32}P-labeled: a virus-containing sample was blotted onto nitrocellulose filters by using a dot blot manifold, baked, and then hybridized with virus-specific probe.

Since B19 encapsidates both positive- and negative-sense strands in equal numbers, B19 DNA forms double-stranded molecules upon extraction and purification (Clewley,

1984). Clewley purified double-stranded B19 DNA and then treated it with nuclease S1 to remove hairpin palindromic loops by cutting at exposed single-stranded regions in the termini. A nearly full-length genomic fragment was cloned by homopolymeric tailing after addition of cytidylic acid tails using terminal transferase. An insertion site was produced by adding guanylic acid tails to the cloning vector pBR322 after its linearization by restriction at the *Pst*I site (Clewley, 1985).

Nonradioactive labels for probes may be used for safety and a long shelf life. For example, Mori and her colleagues used a biotin-labeled DNA probe and streptavidin-alkaline phosphatase conjugate (Mori et al., 1989). A number of investigators have used digoxigenin-labeled probes for detection of B19 (Azzi et al., 1990; Zerbini et al., 1990).

PCR

The PCR offers exquisite sensitivity and the ability to detect B19 DNA in an array of clinical specimens. Primers directed against sequences in the nonstructural protein and the viral capsid protein genes have been used. The high sensitivity of PCR was demonstrated by Clewley (1989), who reported that 60 of 95 anti-B19 IgM-positive serum samples were positive by PCR, while only one was positive by dot blot hybridization. PCR was 99% specific, in that only one sample in a control panel of 100 sera from individuals with other infections was positive by PCR as well as by dot blot hybridization. He concluded that the high rate of detection of B19 DNA by PCR represents a slow rate of decay of viral DNA after peak viremia and is not a clinically significant phenomenon (Clewley, 1989). Koch and Adler, using oligonucleotide primers and detection of amplification products on agarose gels, found PCR to be 10,000 times more sensitive than dot blot hybridization. Southern analysis of amplified product using a radiolabeled oligonucleotide probe complementary to a sequence between the primers is typically 10^3 times more sensitive than dot blot hybridization. PCR has detected B19 DNA in urine, amniotic fluid, pleural fluid, ascites, and leukocyte extracts (Koch and Adler, 1990). PCR also has been used to detect B19 in fetal and adult tissues (Clewley, 1989; Salimans et al., 1989; Naides et al., 1990; Foto et al., 1993; Langnas et al., 1995). PCR has been successfully utilized to diagnose persistent B19 infection in immunocompromised patients, in whom small amounts of B19 DNA in serum may be detected (Frickhofen and Young, 1990; Naides et al.,1993). The utility of attributing clinical syndromes to B19 infection after detection of B19 DNA in tissues by sensitive PCR methods has been questioned. Söderland and colleagues demonstrated B19 DNA in synovial tissue in healthy military recruits undergoing arthroscopy for traumatic knee injuries, using a sensitive PCR-based method. Demonstration of active transcription of viral genes would be necessary to suggest that detected B19 DNA represents active infection, either as a productive infection with virion production or as a restricted infection without virion production but with viral protein production, as demonstrated in hepatocytes (Poole et al., 2004; Poole et al., 2006).

PREVENTION OF AND THERAPY FOR DISEASES DUE TO B19

Parvovirus B19 is ubiquitous, making it difficult to prevent exposure. Community contacts are frequently asymptomatic. However, avoidance of potentially viremic individuals by those at high risk for complications of B19 infection remains a prudent course.

Infection Control

Infection control measures in the outpatient setting are limited to avoiding exposure for high-risk groups. Unfortunately, most infections result from exposure to index cases during the period of viremia, when the index case is either asymptomatic or has nonspecific influenza-like symptoms. In the hospitalized patient, infection control measures are important in avoiding exposure of hospital personnel and patients at risk. Outbreaks of B19 infection among hospital staff have been documented (Bell et al., 1989). Patients with fifth disease should be isolated with secretion precautions until 24 h after onset of rash, arthralgia, or arthritis. Secretion precautions require wearing gowns and gloves when handling body fluids or secretions, such as saliva, nasal aspirates, urine, stool, and blood. Patients who are likely to be viremic are isolated with secretion precautions. However, a potentially viremic patient should not be permitted to share a room with patients with hemolytic anemia, pregnancy, or immune compromise. Isolation of viremic patients should be continued for the duration of the illness and, in most cases, for the entire period of hospitalization. Mothers of infected newborns should also be placed in isolation, under secretion precautions for the duration of the hospital stay. Mothers may visit neonates in the nursery but must follow infection control procedures that include careful hand washing and avoidance of contact with other infants. Household contacts of viremic patients should be isolated with secretion precautions from day 7 until day 18 after contact. Throughout the period of hospitalization, employees, visitors, and patients who are pregnant should be separated from persons who are likely to be viremic (including those with initial fifth disease rash) or who are household contacts of such a patient (Naides, 1989). While these measures have been universally accepted for infection control, some centers also have recommended respiratory precautions for viremic patients (Anderson et al., 1989). Respiratory precautions require separation of the patient in a single room and wearing of a mask by staff during all patient contacts.

Special care should be taken when handling B19 in the laboratory. Laboratory acquisition of infection has been suggested, with the most likely source being aerosolization during centrifugation, resuspension of viral pellets, and washing stages of immunoassays (Cohen et al., 1988). Exposure of B19 virus to ultraviolet light does decrease infectivity, but it also reduces antigenicity of virus preparations used in diagnostic assays. It would appear prudent to survey laboratory personnel for their serological status and to caution individuals who are pregnant or immunocompromised or who have hemolytic anemia about directly working with B19 virus isolates or being in situations that could lead to exposure to the virus.

Therapy

There is no specific antiviral therapy for parvovirus B19. In general, management is symptomatic and supportive. Patients with aplastic crisis usually require transfusion support during the period of areticulocytosis. Adults with chronic B19 arthropathy benefit from nonsteroidal anti-inflammatory drugs that control symptoms of pain and swelling. We have attempted a short course of 5 mg of oral prednisone daily in one patient with B19 arthropathy, without apparent benefit (Naides et al., 1990). When fetal infection is accompanied by severe anemia, transfusion support may be required for fetal survival. Fetuses treated in this manner have survived to term and have been born without apparent long-term sequelae (Peters and Nicolaides, 1990; Soothill, 1990; Sahakian et al., 1991).

We reported one case of B19-induced fetal hydrops without significant anemia, the hydrops in this fetus being due to a viral cardiomyopathy. Digitalization of the fetus in utero resulted in resolution of ascites and other stigmata of congestive heart failure, but the fetus died despite the good initial response (Naides and Weiner, 1989). As described above, individuals with immunodeficiency may develop persistent B19 infection because they lack the ability to produce anti-B19 neutralizing antibodies. Infusion of commercial immune serum globulin, or immunoglobulin, has been used successfully to clear persistent infection in patients with congenital immune deficiency, hematological malignancy, AIDS, or hyper-IgM syndrome (Kurtzman et al., 1988; Kurtzman et al., 1989a; Frickhofen et al., 1990; Seyama et al., 1998; Blaeser et al., 2005). Intravenous immunoglobulin is given at a dose of 0.4 g/kg of body weight daily for 5 or 10 days. Therapy results in clearing of B19 viremia and improvement in bone marrow suppression (Frickhofen et al., 1990). As an alternative to the intravenous route, we have given an AIDS patient, with chronic anemia and a monthly transfusion requirement, serum immune globulin intramuscularly with good initial response. Prior to treatment, reticulocytes were undetectable and the anemia did not respond to erythropoietin. After initial intramuscular immunoglobulin injection, the reticulocyte count increased and the transfusion requirement decreased (Naides et al., 1993). While intramuscular immune serum globulin has the advantage of ease of administration and significantly decreased costs, efficacy of the intramuscular route compared to intravenous immune serum globulin administration remains to be determined.

HBOV

Using a sequential method for host DNA depletion, random PCR amplification, and molecular screening, Allander and colleagues identified two isolates of a novel virus in respiratory secretions collected in a clinical laboratory. The new virus was homologous to bovine parvovirus and minute virus of canine, members of the recently defined genus, bocavirus (*bo*vine parvovirus and minute virus of *ca*nine; hence, *boca*virus). This human bocavirus (HBoV) contained three open reading frames encoding nonstructural protein NS1, another nonstructural protein of unknown function (NP-1), and the capsid proteins VP1 and VP2. Individuals with HBoV-positive sputa were hospitalized infants and children with a lower respiratory tract infection. Chest roentgenograms typically showed bilateral interstitial infiltrates (Allander et al., 2005). Symptoms include fever, cough, and sore throat (Bastien et al., 2006). Detection of HBoV in respiratory samples peaks in the winter months and is seen worldwide (Arnold et al., 2006; Weissbrich et al., 2006; Manning et al., 2006; Pozo et al., 2007). Absence of HBoV in respiratory secretions from healthy individuals has provided further evidence for HoBV pathogenicity (McIntosh, 2006; Kesebir et al., 2006; Manning et al., 2006). HoBV was reportedly present in 9.1% of patients in Spain with gastroenteritis, compared to 7.7% with respiratory infection, a finding confirmed by a group in Hong Kong (Vicente et al., 2007; Lau et al., 2007). Prevalence of antibodies to VP1 in a Japanese population was lowest in the age group of 6 to 8 months and gradually increased with age; all children tested were seropositive by the age of 6 years, with an overall seropositivity rate of 71.1% in the group up to 41 years of age (Endo et al., 2007). Identification of HBoV in 5 of 16 Kawasaki disease patients prompted the suggestion that HBoV may play a role in the pathogenesis of that idiopathic disease (Catalano-Pons et al., 2007). Coinfection with other viruses varies, but the rate of adenovirus coinfection was reported to be as high as 69.2% in one study (Hindiyeh et al., 2008). HBoV may persist or reactivate in immunocompromised patients, as reported for three children with acute lymphoblastic leukemia (Koskenvuo et al., 2008). The full spectrum of HBoV disease, including its role in asthma, remains to be elucidated.

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Measles, Mumps, and Rubella

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31

MEASLES VIRUS

Background

Humans are the only known natural host for measles (rubeola) virus. Enders and Peebles first reported the successful isolation of measles virus in human and rhesus monkey kidney tissue cultures in 1954 (Enders and Peebles, 1954). At that time there were more than 400,000 cases of measles reported each year in the United States. However, since virtually all children would acquire measles, the true number probably exceeded 3 million per year. In 1963, both an inactivated and a live attenuated vaccine (Schwartz, 1962) were licensed for use in the United States. The killed vaccine eventually proved less effective, and children who received this material were at risk of developing an atypical severe form of the disease when subsequently exposed to live measles virus. In 1967, the inactivated vaccine was discontinued. A live, further attenuated vaccine (Moraten strain) was licensed in 1968 and is the vaccine used currently in the United States. Initially, vaccine was administered to children over 9 months of age, but it became apparent that young infants did not mount an adequate immune response. Thus, the recommended age of vaccination was moved to between 12 and 15 months of age. A resurgence of measles disease occurred in the United States between 1989 and 1991, with over 55,000 reported cases and over 100 deaths. This was due to a small number of vaccine failures in the school age population and low vaccination coverage in more populated areas (Atkinson and Orenstein, 1992). It is now recommended that vaccination be initiated at age 12 to 15 months, with a second dose administered anytime from 1 month post-first dose to before entry into preschool, in combination with mumps and rubella vaccines (MMR). Measles remains endemic to most areas of the world, but the use of vaccination strategies that include a second opportunity for measles vaccination have resulted in a drastic reduction in measles mortality (Wolfson et al., 2007). Measles is no longer endemic to the United States (Katz and Hinman, 2004), but the threat of reintroduction from areas of endemicity requires vaccination coverage at or near 95% and disease surveillance capable of detecting imported measles cases that have the potential to initiate outbreaks in susceptible groups (Centers for Disease Control and Prevention [CDC], 2004; CDC, 2008c).

Characteristics of Virus

Measles virus is a single-stranded, negative-sense RNA virus and the prototypic member of the *Morbillivirus* genus of the *Paramyxovirinae* subfamily of the family *Paramyxoviridae*. The measles virion is spherical, with a diameter ranging from 120 to 250 nm. It has an envelope composed of glycoproteins, the hemagglutinin (HA) and fusion proteins, and lipids. The proteins appear as short surface projections and are responsible for receptor binding and virus entry into susceptible cells. The matrix protein is positioned under the virion envelope and, unlike the surface proteins, is neither glycosylated nor transmembranous. The envelope encloses an elongated helical nucleocapsid in which protein units are spirally arranged around the nucleic acid. The nucleoprotein, phosphoprotein, and large polymerase protein, in conjunction with the virion negative-strand RNA, comprise the ribonucleoprotein complex, the replicating and transcriptional unit of measles virus (Lamb and Kolakofsky, 1996).

Clinical Aspects

Measles is spread through direct contact with infected droplets originating from a cough or sneeze or from contaminated fomites. It is a highly contagious, acute biphasic disease with a prominent prodrome preceding the exanthemic phase. Susceptible persons intimately exposed to a measles patient have a 99% chance of acquiring the disease. Prior to the use of vaccines, more than 90% of the population had measles before 10 years of age. After an incubation period of 9 to 11 days, there is an initial 3- to 4-day prodromal period characterized by fever, cough, coryza, and conjunctivitis. The incubation period in adults may last up to 3 weeks. A fever occurs 24 h or less before other symptoms appear, and these increase in severity, reaching a peak with the appearance of the rash on the fourth or fifth day.

Bluish-white lesions with a red halo, Koplik spots, appear on the buccal or labial mucosa in 50 to 90% of the cases, 2 to 3 days after the onset of the prodrome. These lesions are small, irregular red spots with bluish-white specks in the centers and are located on the inner lip or opposite the lower molars and are pathognomonic for measles (Koplik, 1962). They may be few in number early in the prodrome; however, they increase rapidly to spread over the entire surface of the mucous membranes. A lesion somewhat similar in appearance to Koplik spots has been reported with ECHO-9

(Coxsackie A23) and Coxsackie A16 and A9 virus infections. The measles rash is first evident behind the ears or on the forehead. The lesions are red macules, 1 to 2 mm in diameter, which become maculopapules over the next 3 days. By the end of the second day, the trunk and upper extremities are covered with rash, and by the third day, the lower extremities are affected. The rash resolves in the same sequence, lasting approximately 6 days. The lesions turn brown and persist for 7 to 10 days and then are followed by a fine desquamation.

Complications

The most frequent complication of measles involves infections of the lower respiratory tract. Croup, bronchitis, bronchiolitis, and rarely, giant-cell interstitial pneumonia may occur. Otitis media is a common bacterial complication of measles. Prior to the advent of antibiotics, these complications contributed to a high number of fatalities and significant morbidity. Excluding pneumonia and otitis media, the most frequent serious complication of measles is postinfectious encephalitis. It occurs in 0.1 to 0.2% of measles patients during any stage of the illness, although it is most common 2 to 7 days after the onset of the exanthem. Death occurred in 1 to 2 of every 1,000 reported cases in the United States. Other complications include thrombocytopenic purpura, appendicitis, myocarditis, and mesenteric lymphadenitis (Gershon and Krugman, 1979).

Subacute sclerosing panencephalitis (SSPE), also called Dawson's encephalitis, is a persistent measles infection of the central nervous system (CNS). SSPE is a progressive, invariably fatal, encephalopathy characterized by personality changes, mental deterioration, involuntary movements, muscular rigidity, and death. It usually begins 4 to 10 years after the patient has recovered from naturally acquired measles. Successful isolation of measles virus from the brain and lymphoid tissues of SSPE patients (Horta-Barbosa et al., 1969; Horta-Barbosa et al., 1971) clearly established the etiologic agent involved. In the prevaccine era, the incidence of SSPE was approximately 1:100,000 to 1:1,000,000 cases, although recent studies suggest that the incidence may have been about 19-fold greater (Bellini et al., 2005). The introduction of live attenuated measles vaccine raised concerns that the vaccine virus might cause SSPE, but epidemiologic studies demonstrated a dramatic decrease in the frequency of this disease (Halsey et al., 1980). Recent studies have clearly demonstrated that measles vaccine virus is not involved in the genesis of SSPE and that the use of measles vaccine not only is beneficial in preventing acute measles but has all but eliminated SSPE from the United States (Bloch et al., 1985; Jin et al., 2002; Bellini et al., 2005; Campbell et al., 2007).

Transplacental infections have been associated with some fetal effects. There is an apparent increased frequency of premature labor, low birth weight, abortions, and stillbirths (Eberhart-Phillips et al., 1993). The teratogenic potential of gestational measles has been neither proved nor refuted (Fuccillo and Sever, 1973; South and Alford, 1980).

Atypical measles occurred in some children previously vaccinated with killed measles virus vaccines when they became infected with wild measles (Fulginiti et al., 1967). Fever, a prodromal period, and subsequent rash characterized the disease. During the prodrome, some patients experienced malaise, myalgia, headache, nausea, and vomiting. Symptoms usually lasted for 2 to 3 days, and individuals frequently had a sore throat, conjunctivitis, and photophobia along with nonproductive cough and pneumonia. Chest X rays often showed patchy infiltrates. The rash produced was different from that of typical measles. It could be a mixture of macules, papules, vesicles, and pustules. Frequently, there was a petechial component, which began at the distal extremities and concentrated on the hands, wrists, ankles, and feet and then progressed centrally toward the trunk. Koplik spots were not reported, and the face was rarely involved. Edema often occurred in the extremities. The appearance of atypical measles could be confused with Rocky Mountain spotted fever.

Among immunocompromised patients, measles can be severe and prolonged. This is particularly a risk for patients with certain leukemias, lymphomas, or human immunodeficiency virus infection (Markowitz et al., 1998).

Laboratory Diagnosis

Overview

While the diagnosis of a case of measles can be made based on observation of clinical signs and symptoms, expedient laboratory confirmation is extremely important because of possible confusion with other rash-causing illnesses. The incidence of measles has decreased in many areas to the extent that medical personnel may be unfamiliar with the clinical presentation of the disease. Moreover, milder forms of measles have been reported to occur in previously vaccinated individuals (Edmonson et al., 1990), and in many instances, these patients do not meet the clinical case definition. Because of the highly infectious nature of measles virus, it is important that laboratory confirmation of suspected cases be completed as quickly as possible so that control measures can be initiated. Therefore, the preferred laboratory diagnostic procedure is the detection of measles-specific IgM antibody in a single serum sample obtained during the acute phase of the disease (Tuokko, 1984; Helfand et al., 1997). There are a number of sensitive and specific commercial enzyme immunoassay (EIA) kits available, which are relatively easy to perform and give results within a few hours. Immunoglobulin M (IgM) can also be detected using indirect immunofluorescence antibody (IFA) tests, but this method is not as sensitive as EIA, and is prone to producing erroneous results.

Other diagnostic serological methods include hemagglutination inhibition (HI) and plaque reduction neutralization test (PRNT) assays, but these tests are time-consuming and require that both acute- and convalescent-phase serum samples be available for testing. At present, it is desirable to obtain the necessary specimens when the patient is first seen, since in many regions of the world, convalescent-phase serum specimens may never be obtained. Finally, laboratory confirmation of measles can be made by detecting viral antigens or RNA using either indirect IFA or reverse transcription (RT)-PCR.

Serologic Diagnosis

Traditional antibody tests such as HI, PRNT, and EIA have been used extensively in the serologic diagnosis of measles. However, because of the availability of sensitive and specific commercial kits, EIAs have become the most widely used test format. Commercial EIAs also have the ability to measure measles-specific IgM as well as IgG responses and therefore have particular importance in measles diagnosis as well as measles control programs. Some of the available kits were found to have sensitivities and specificities that compared favorably with PRNT (Ratnam et al., 1995; Cohen et al., 2006).

Standard EIAs

Production of measles antigen for EIA has traditionally been done using tissue culture-adapted strains (e.g., Edmonston strain) grown in Vero cells. Measles is a highly cell-associated virus, so infected cells must be disrupted by freeze-thaw or sonication before the clarified cell lysates are used as antigens. Measles antigens have been produced in recombinant DNA expression systems, such as baculovirus and yeast (Hummel et al., 1992; Slibinskas et al., 2004). EIAs using the baculovirus-expressed nucleoprotein (MVN) in both capture and indirect formats had high levels of sensitivity and specificity compared to plaque neutralization and other commercial EIAs (Hummel et al., 1992). The baculovirus system has the added advantage that the yields of antigen possible in the insect cell culture system far exceed those of virus growing in mammalian cells. Also, the necessity of handling large amounts of a virus that is potentially pathogenic for humans is avoided.

To detect IgG, indirect EIA has been successful (Erdman et al., 1991). For this indirect test, either whole-virus antigen diluted in 0.05 M bicarbonate buffer (pH 9.5) or recombinant antigen diluted in phosphate-buffered saline (PBS) is placed into polystyrene microtiter plates. Serum specimens are diluted in PBS containing 4% normal goat serum (plus 4% *Spodoptera frugiptera* [Sf9] cell lysate for recombinant antigen) and 0.05% EDTA and are then added to the washed plates. Bound antibody can be detected with standard commercial reagents such as goat anti-human IgG conjugated to either alkaline phosphatase or biotin. The assays are developed with the appropriate substrate, and the plates can be read either by eye or with the aid of a spectrophotometer.

For the detection of measles-specific IgM in single, acute-phase serum samples, commercial kits based on both indirect and IgM capture formats have been used (Erdman et al., 1991; Mayo et al., 1991; Samuel et al., 2003; Slibinskas et al., 2004). Though IgM capture is generally regarded as the more sensitive format, some of the commercial indirect EIA kits have sensitivities and specificities that approached those of the capture format (Arista et al., 1995; Ratnam et al., 2000). The availability of excellent immunoabsorbants capable of removing IgG from diluted serum specimens is largely responsible for the success of the indirect IgM methods.

Interpretation of EIA Results

The occurrence of clinically defined measles in persons, years after receiving vaccine, has been attributed to primary vaccine failure due to insufficient primary antigenic stimulation as well as secondary vaccine failure due to a putative loss of protective antibody or waning immunity. The use of sensitive EIAs for detection of measles IgM antibody has provided a better description of primary and secondary antibody responses in persons following primary and secondary vaccination and natural measles virus infection (Erdman et al., 1993). The findings of these studies indicated that (i) an IgM response follows primary measles vaccination or measles infection in the immunologically naive, (ii) an IgM response is absent upon revaccination of those previously immunized, and (iii) an IgM response may follow clinical measles virus infection independent of prior immunization status. There is also evidence that case contacts who have a history of natural infection or vaccination and have a resident IgG response may also develop a secondary IgG or an IgM response to currently circulating virus (Muller et al., 1996; Helfand et al., 1998). These close contacts may show few, if any, of the clinical signs of measles infection. Table 1 summarizes the possible interpretations of EIA results.

The time at which the serum specimen is collected may affect the results of the capture-IgM EIA. It was possible to detect measles virus-specific IgM in 77% of serum specimens taken from measles patients during the first 72 h after rash onset, while 100% of specimens taken 4 to 11 days after rash onset contained detectable IgM. Therefore, some false-negative reactions may occur if samples are taken within 72 h after rash onset, requiring that a second serum sample be collected (Helfand et al., 1997).

New EIAs

A potentially promising development has been the use of EIAs to measure the avidity of IgG antibodies to measles (Tuokko, 1995; Narita et al., 1997; de Souza et al., 1997). As the immune response matures, low-avidity antibodies are replaced with high-avidity antibodies. These avidity differences can be detected by using protein denaturants, typically 8 M urea, or diethylamine in the washing step of the

TABLE 1 Interpretation of measles EIA results[a]

IgM result	IgG result	Previous infection history	Current infection	Comments
+	+ or –	Not vaccinated, no history of measles	Recent first MMR	Seroconvert[b]
+	+ or –	Not vaccinated, no history of measles	Wild-type measles	Seroconvert[b]; classic measles
+	+ or –	Previously vaccinated, primary vaccine failure	Recent second MMR	Seroconvert[b]
–	+	Previously vaccinated, IgG$^+$	Recent second MMR	IgG level may stay same or rise
+	+	Previously vaccinated, IgG$^+$	Wild-type measles	May have few or no symptoms[c]
+	+	Recently vaccinated	Exposed to wild-type measles	Cannot distinguish if vaccine or wild type; evaluate on epidemiologic grounds[d]
+ or –	+	Distant history of measles	Wild-type measles	May have few or no symptoms[c]; if clinically compatible, may have been misdiagnosed initially

[a] +, positive; –, negative.
[b] IgG response depends on timing of specimen collection (Helfand et al., 1997).
[c] If so, do not consider contagious unless clinical presentation is consistent with measles.
[d] If IgM negative, helpful to rule out wild-type measles infection.

indirect EIA for measles IgG. An avidity index is then calculated by comparing the optical densities obtained with and without the denaturant in the wash buffer. These tests have been able to successfully differentiate between primary and secondary responses to vaccination and to natural infection (Paunio et al., 2000; Pannuti et al., 2004).

The HI Test

Because of the availability of EIAs, the HI test is now more useful as a research tool than as a diagnostic test. The HI test measures antibodies to the measles HA and has a sensitivity equivalent to that of EIA. The HI test can be performed in a few hours but requires special reagents and paired serum samples. Other limitations of the HI test are the requirement for fresh vervet monkey red blood cells (RBC), the difficulty of producing sufficient antigen, and the possible presence of nonspecific inhibitors of hemagglutination in serum. Serum should be inactivated at 56°C for 30 min before testing. Nonspecific inhibitors may be removed by absorption of the serum with an equal volume of 25% (wt/vol) kaolin in PBS. RBCs should be obtained from monkeys lacking measles antibody, and RBCs from some animals demonstrate HA better than those from other animals. It is necessary to preselect donor animals for a strong positive HA with the measles antigen. RBCs can be stored in Alsever's solution at 4°C for up to 1 week. On the day of the test, RBCs are washed three times in PBS and resuspended in PBS at 0.5%. Viral antigen is propagated in Vero cells, BSC-1, lung fibroblasts, or dog kidney. When cytopathic effect (CPE) approaches 100%, the cultures are frozen and thawed three times. Tween 80 is added to a concentration of 0.125%, mixed at 4°C, and then centrifuged at 1,000 × *g* for 20 min. The supernatant is saved as antigen. The choice of measles strain to use as the antigen is important, since many wild-type viruses do not agglutinate RBCs. More-consistent results will be obtained using the Edmonston strain from the ATCC.

Titer of the HA is determined as follows. Twofold dilutions of antigen in PBS in a volume of 0.025 ml are added to microtiter V-bottom plates. To each well, 0.025 ml of 0.5% RBC is added. The plate is shaken briefly and incubated at 37°C for 30 min to 1 h. The highest dilution of antigen giving complete HA is the endpoint and contains 1 HA unit. To perform the test, serial twofold dilutions of serum specimens in a volume of 0.025 ml are added to microtiter wells. An equal volume of antigen diluted in PBS to contain 4 HA units is added, and the mixtures are incubated for 1 h at 37°C. Then 0.05 ml of 0.5% RBC suspension is added, shaken, and allowed to settle at 37°C for 1 to 2 h. Titers are recorded as the highest serum dilution resulting in complete HI.

The PRNT

The PRNT, which measures neutralizing antibodies that are directed against the surface glycoproteins of measles virus, is more sensitive than HI or EIA (Albrecht et al., 1981). Since functional antibodies are being detected, the PRNT provides the best serologic correlate for the assessment of immune protection. However, the PRNT is not suitable for routine serologic diagnosis because it is very labor-intensive, requires paired serum samples, and takes 5 to 7 days to perform. Recently a World Health Organization (WHO) working group standardized the PRNT for use in aerosol vaccination studies, so the results of those assays could be compared between and among studies (Cohen et al., 2007).

In the PRNT, measles-specific antibody in serum combines with and neutralizes measles virus, preventing it from infecting a cell monolayer and forming a plaque under the overlay. The endpoint for the test is the highest dilution of serum which will reduce the number of plaques by 50%. Serum dilutions are made in 96-well microtiter trays proceeding either in twofold or fourfold steps (depending on the expected titer of the serum). Once the dilutions of serum samples are made, an equal volume (120 µl) of a dilution of virus containing 25 to 35 PFU is added to each well and the mixtures are incubated for 2.5 h at 36°C. After incubation, 100 µl of the serum-virus mixture is added to each of two 16-mm tissue culture plates containing Vero cell monolayers. These trays are then incubated for 1 h at 36°C. After the incubation, the inoculum is removed by aspiration and the monolayers are covered with overlay medium consisting of either 2% carboxymethylcellulose in Leibovitz-15 medium or 1% agarose in Eagle's medium. The trays are then incubated for 5 days at 36°C. On day 4, the trays are stained with a solution of neutral red in cell culture medium. On day 5, the overlay is removed and the plaques are counted. Plaque counts in the two wells representing a given serum dilution are averaged. A WHO standard serum with a known neutralizing titer is run as a standard with each assay.

Direct Examination of Specimens for Viruses and Viral Antigens

Virus Isolation

Though other cell lines have traditionally been used, an Epstein-Barr virus (EBV)-transformed, B lymphoblastoid cell line, B95a, is the preferred cell line for primary isolation of measles virus (Kobune et al., 1990). These cells are up to 10,000 times more sensitive for isolation of measles virus from clinical specimens than other cell lines such as Vero and primary monkey kidney. B95a cells are relatively easy to maintain in the laboratory, and the measles CPE is readily observed. However, laboratorians should note that this cell line does produce EBV and should be handled as infectious material at all times.

When cultured in Dulbecco's modified minimum essential medium supplemented with 100 U of penicillin/ml, 100 µg of streptomycin/ml, and fetal bovine serum (FBS), these cells will adhere to the surface of the culture vessel, and the adherent cells are referred to as B95a. Cell growth is sustained in medium containing 8 to 10% FBS. FBS is used at a 2% concentration for cell maintenance during viral isolation. Cell stocks can be prepared using standard cryoprotection medium. Unfortunately, a commercial source for this cell line is no longer available.

B95a cells should be at 85% confluency when inoculated with specimens. After inoculation with clinical material, the cells should be maintained in Dulbecco's modified minimum essential medium plus 2% FBS and antibiotics. Change the medium every 3 to 5 days, and passage the cells by splitting at 1:2 every 5 to 7 days. Check for viral CPE (syncytium formation) daily. When CPE is visible, continue to feed the cells until the CPE becomes extensive. When CPE is visible over at least 75% of the monolayer, viral stocks can be prepared by freezing the cells at –70°C. It is advisable to perform 3 to 4 blind passages before discontinuing efforts for viral isolation.

The Vero/SLAM cell line has been recommended for use in the WHO laboratory network. These cells are the result of the transfection of Vero cells with a plasmid encoding the gene for the human signaling lymphocyte activation molecule (SLAM) molecule (Ono et al., 2001). SLAM has been shown to be a receptor for both wild-type and laboratory-adapted

strains of measles. Testing conducted to date indicates that the sensitivity of Vero/SLAM cells for isolation of measles virus is equivalent to that of B95a cells. The advantage to the Vero/SLAM cells is that they are not persistently infected with EBV and, therefore, are not considered hazardous material like B95a cells. This provides a significant safety advantage for laboratorians and greatly facilitates international shipments. The disadvantage of the Vero/SLAM cells is that they must be cultured in medium containing geneticin (G418) to retain SLAM expression, thus increasing the cost of the cell culture medium. This cell line is available from the CDC Measles, Mumps, and Rubella Laboratory Branch upon request.

Cytologic Examination

Intranuclear and intracytoplasmic inclusions and giant cells are characteristic CPE for cells infected with measles virus. Cytologic examination of various tissue specimens and secretions for these Warthin-Finkeldey giant cells can be used as a diagnostic procedure. Secretions are obtained by aspiration of mucus from the nose or by swabbing the nasal mucosa with a sterile, cotton-tipped applicator. Slides can be stained with either Wright stain or hematoxylin and eosin. Tissue samples may be fixed in 10% formalin, embedded in paraffin, sectioned, and then stained with hematoxylin and eosin stain (Gershon and Krugman, 1979). Staining of tissue specimens with monoclonal antibodies to the measles nucleoprotein has been used to support the diagnosis of giant-cell pneumonia, measles inclusion body encephalitis, and SSPE (Zaki and Bellini, 1997; Bellini et al., 2005).

Immunofluorescence

Detection of measles virus can be achieved using an indirect IFA to examine clinical specimens as well as cell cultures infected with clinical material. The standard assay uses a commercially available monoclonal antibody to the nucleoprotein of measles virus and fluorescein-conjugated goat anti-mouse antiserum. Nasal secretions should be centrifuged at 800 × *g* to pellet the cells. The cell pellets are then washed several times with sterile saline before being applied to a glass slide and fixed in cold, 80% acetone for 10 min at –20°C (Minnich et al., 1991; Smaron et al., 1991).

Standard and Real-Time RT-PCR

Standard and real-time RT-PCR assays have been used in research settings to detect measles virus RNA in clinical specimens and infected cells (Nakayama et al., 1995; Rota et al., 1995; Hummel et al., 2006). While serologic testing is recommended for routine diagnosis of acute measles infections, there are circumstances where the use of molecular methods of detection is more advantageous. Therefore, RT-PCR should be considered for diagnostic use where IgM testing is compromised by the concurrent or recent use of measles-containing vaccine as part of outbreak response or in settings of high vaccine coverage (Hyde et al., 2006). Likewise, molecular detection methods can be used when cell culture isolation is not a practical alternative and/or when genetic characterization of the virus is required. RT-PCR has been particularly useful for the laboratory confirmation of measles inclusion body encephalitis, SSPE, and giant-cell pneumonia. Sequence determination obtained from the PCR products in conjunction with phylogenetic analysis has proven useful in suggesting the possible source of virus involved in outbreaks, tracking transmission pathways during outbreaks, and differentiating between vaccine and wild-type strains of measles (Bellini and Rota, 1998).

RT-PCR techniques have had the greatest impact on molecular surveillance activities. Measles RNA is easily amplified from RNA extracted from infected cell culture, and it has been possible to detect measles RNA in nasal, urine, and oral fluid, dried blood spots (Katz et al., 2002), and sometimes serum samples by RT-PCR even when virus isolation has been unsuccessful (Jin et al., 1997; Oliveira et al., 2003). Most recently, real-time RT-PCR and semi-quantitative molecular assays such as loop-mediated isothermal and quantitative PCR methods have been developed for measles using various target genes along the measles genome (El Mubarak et al., 2005; Plumet and Gerlier, 2005; Hummel et al., 2006). Several of these assays claim sensitivities of 10 to 50 RNA copies.

Control and Prevention

Individuals having an illness compatible with measles should be cared for in such a way that contact with other people or patients is minimal. The communicability of measles virus is extremely high. Therefore, any susceptible individuals who had direct face-to-face contact with the infectious individual should obtain prophylactic treatment. Risk, other than face-to-face, is very low, and therefore, postexposure prophylaxis is unnecessary. Measles vaccination may provide protection if given within 72 h of exposure (American Academy of Pediatricians, 1997). The Immunization Practices Advisory Committee supports readmission to school of all previously unimmunized children immediately following vaccination (CDC, 1989). Immune globulin, given within 6 days of exposure, can prevent or modify measles virus infection. It is indicated for susceptible, close contacts of measles patients, particularly if they are less than 1 year of age. If immune globulin is used for a child at this age, measles vaccine should be given 5 or 6 months later provided that the child is at least 12 months old.

After a further attenuated variant of the Edmonston B vaccine was introduced in 1968, the reported cases of measles took a dramatic downward turn. In 1960, the cumulative total number of cases was 399,852 from week 1 to 35. In 1970, the total was 39,365; in 1981, it was 2,562; in 1982, it was 1,188; and in 1983, for the same period, the total number of cases was 1,194 (CDC, 1982). There was hope that 1983 would be the year in which measles would be eliminated from the United States, but this goal was not accomplished. In fact, reported cases increased every year until 1986, when there were 6,282 cases. A major resurgence of measles occurred between 1989 and 1991 and led to the current 2-dose MMR schedule currently in place. Epidemiologic and laboratory data suggest that the transmission of indigenous measles was interrupted in the United States in 1993 (Watson et al., 1998; Rota et al., 1996). Since the full implementation of the 2-dose MMR vaccination schedule, measles cases decreased to less than 100 cases since 2000 and have been even lower in last few years. In 1993, the Childhood Immunization Initiative called for the elimination from the United States by 1996 of indigenous transmission of measles, rubella, congenital rubella syndrome, and three other childhood diseases. Endemic transmission of measles was interrupted in the United States in 1992, and measles was declared eliminated from the United States in 2000 (Katz and Hinman, 2004). Imported cases of measles from areas of endemicity continue to be a cause for concern, particularly when such cases come in contact with unvaccinated groups (Parker et al., 2006; CDC, 2008b). Vaccination coverage must be maintained at a very high level to sustain the elimination status of the United States.

MUMPS VIRUS

Background

Mumps virus infection was probably first described around the fifth century B.C. by Hippocrates. The name "mumps" is thought to be derived from the mumbling speech of patients afflicted with this disease. Johnson and Goodpasture (1934) introduced saliva from patients with mumps directly into the Stensen's duct of rhesus monkeys and induced parotitis; they identified the etiologic agent as a virus. Mumps virus was first isolated in the amniotic cavity of a chicken embryo in 1945 (Habel, 1945). The virus was later isolated and passaged in monkey kidney cells and HeLa cells from mumps patient clinical specimens including saliva and cerebrospinal fluid (CSF) (Henle and Deinhardt, 1955). Although a killed version of the mumps vaccine was first developed, the first and most successful live-attenuated mumps vaccine (Jeryl-Lynn) was developed in 1966 by passage of the virus in chicken embryo cell cultures (Buynak and Hilleman, 1966). This live-attenuated vaccine remains the only mumps vaccine licensed in the United States and is used in combination with measles and rubella vaccine as a component of the MMR vaccine.

Characteristics of the Virus

Mumps virus is a member of the *Paramyxoviridae* family, the *Paramyxovirinae* subfamily, and the *Rubulavirus* genus of single-stranded, negative-sense, enveloped RNA viruses. Virus particles are pleomorphic but generally spherical structures and range in size from 85 to 300 nm in diameter (Cantell, 1961). Filamentous structures have also been observed. The mumps genome is encapsidated by nucleoprotein, and as in the case of measles virus, the phosphoprotein and polymerase are associated with the encapsidated RNA to comprise the ribonucleoprotein complex. The envelope contains an HA-neuraminidase and a fusion (F, hemolysin) protein as well as a matrix protein and a short hydrophobic (SH) membrane-associated protein (Wilson et al., 2006). The gene encoding the SH protein is highly variable and has been used as the basis of genotyping mumps viruses for molecular epidemiological purposes (Jin et al., 1999; Jin et al., 2005; Muhlemann, 2004).

Clinical Aspects

Humans are the only known host and reservoir of mumps virus. The infection can be either clinically apparent or subclinical. Infection is endemic worldwide, usually affecting the 6- to 10-year-old age group in unvaccinated populations; it occurs predominantly in the spring. About 30 to 40% of mumps infections produce parotitis, 20 to 30% of infections are asymptomatic, and 50% are associated with nonspecific or respiratory symptoms. The average incubation period is 16 to 20 days. Parotitis occurs more frequently in children (Philip et al., 1959). The parotitis is sudden and may not be preceded by any prodromal symptoms. Swelling of the glands reaches a maximum after 48 h, and they usually remain swollen for a period of 7 to 10 days. There may be little or no increase in body temperature. Approximately 20 to 30% of postpubertal men acquiring mumps develop epididymo-orchitis between 1 and 2 weeks following the parotitis; however, sterility is not a common sequela of infection and only 1 to 12% of the cases are bilateral.

Another complication of mumps virus infection is meningoencephalitis, which has an incidence from 5 to 10%. Encephalitis is one CNS complication, but mumps virus infection has been linked to other rare CNS complications such as transverse myelitis, cerebellar ataxia, poliomyelitis-like syndrome, and Guillain-Barré syndrome. About 5% of adult females with mumps may develop oophoritis. Other complications, such as pancreatitis, thyroiditis, neuritis, inflammation of the eye, and inner ear infection, can be encountered. There have been reports of diabetes mellitus being associated with mumps, but this remains inconclusive (Sultz et al., 1975; Ratzmann et al., 1984).

Seroepidemiologic surveys have indicated that 80 to 90% of adults have evidence of prior exposure to mumps, either through vaccination or mumps infection. Mumps is transmitted by saliva containing the virus either by direct transfer, air-suspended droplets, or recently contaminated fomites. Approximately 85% of susceptible contacts can become infected when first exposed, and 25 to 30% of the infections may be asymptomatic. The virus is thought to multiply in the upper respiratory tract and local lymph nodes, then invade the bloodstream, and finally affect the salivary glands and other organs. About 18 days elapse between the time of exposure and the first detectable enlargement of the salivary glands. The incubation period may range from 14 to 24 days. The period of communicability can be from 7 days before the salivary gland involvement until 9 days thereafter. The virus is also excreted in the urine for as long as 14 days after onset of illness. Mumps infections in previously vaccinated individuals likely result in decreased levels of virus shedding into the buccal cavity (Okafuji et al., 2005). This and compliance issues with the current 9-day isolation period has led to considerations by CDC and other public health agencies to reduce the number of days of patient isolation from 9 days to 5 days post-symptom onset (Bitsko et al., 2008; CDC, unpublished data).

Laboratory Diagnosis

Clinical diagnosis of mumps infection can be made reliably when typical parotitis is evident at the time of patient examination. However, since parotitis may be caused by other viral and nonviral diseases or conditions, and as much as 25 to 30% of mumps infection can be asymptomatic, diagnosis by viral isolation, molecular detection, or serological techniques is preferable. Before a major 2006 mumps outbreak in the Midwest (CDC, 2006a, 2006b, 2006c; Dayan et al., 2008), the incidence of mumps in the United States had decreased by more than 99% in association with the advent of the MMR (MMRII) vaccine and especially with the recommendation of 2 doses of the MMR vaccine before school entry resulting from a measles resurgence in 1989 (CDC, 1989). The laboratory criteria for the diagnosis of mumps are (i) isolation of the mumps virus in cell culture from clinical specimens; (ii) molecular detection of mumps RNA; (iii) a significant rise between acute- and convalescent-phase titers in serum, using a quantitative measure of antibody; or (iv) a positive IgM response in an EIA when only a single specimen is available.

Isolation of Virus

Virus isolation from saliva, blood, urine, or CSF confirms the presence of recent mumps infection (Utz et al., 1957; Utz et al., 1958). While primary monkey kidney cell cultures are likely the most sensitive cells, availability, risk of infection with herpes B virus, and animal rights issues have resulted in the use of continuous cell lines. Growth of mumps virus has been noted to occur in primary human cell cultures (Hopps and Parkman, 1979); continuous cell lines such as HeLa and Vero are currently the cell lines of choice. Frequently, these cell lines are used in shell vial culture to

promote more expedient and efficient detection of mumps virus (Germann et al., 1998; Reina et al., 2003). Although mumps virus infection results in characteristic CPE consisting of large syncytia, some isolates vary in the intensity and frequency of the CPE and thus must be confirmed by IFA staining (Swierkosz, 1995; Reina et al., 2003), by immunocytometric assay (Chen et al., 2007), or by molecular detection methods, such as RT-PCR (Boriskin et al., 1993; Palacios et al., 2005).

Mumps virus is stable for several days at 4°C. Stability increases with decreasing temperature, and the virus may be stored for months or longer at –70°C. Most viral transport media contain 1 to 2% protein, which greatly improves the stability of mumps virus. Throat swabs, urine, and CSF specimens should be inoculated onto susceptible cells within a few hours following sample collection. Likewise, specimens collected for RNA extraction for molecular assays, such as RT-PCR or real-time RT-PCR should be processed within a few hours.

Conventional Serology

Many serological assays have been used to detect mumps antibodies. HI, complement fixation (CF), and neutralization (NT) are standard methods for mumps virus detection and supply reproducible results. The serological confirmation of mumps using any of these methods requires a fourfold difference in serial dilution endpoint titration between acute- and convalescent-phase serum specimens collected 2 to 3 weeks apart. All three methods primarily measure IgG, and the interpretation of results can be confounded by cross-reactivity with parainfluenza virus antibodies. These assays have been described in detail elsewhere (Hopps and Parkman, 1979) and are not likely to be used routinely in the clinical laboratory. Due to the enhanced sensitivity, high throughput, relative ease, and low cost, enzyme-linked immunosorbent assays are the serological assays of choice (Tuokko, 1984; Glikmann et al., 1986; Berbers et al., 1993).

Enzyme-linked immunosorbent assay kits are widely available commercially. These assays use a partially purified mumps antigen bound to a solid support such as the wells of a microtiter plate. IgM assays formatted in this way must provide a means to remove potentially interfering mumps IgG from the assay. Historically, these indirect IgM assays were vulnerable to the presence of rheumatoid factor in serum as a source of false-positive reactions. Capture IgM formats from several commercial sources are currently available, and they circumvent the interference of rheumatoid factor. These assays vary greatly in sensitivity (range, 24 to 51%), and the best specificity measured was 82% (Krause et al., 2007). Of considerable interest is the use of oral fluid rather than serum in the determination of IgM for acute mumps infections and IgG for immune status for measles, mumps, and rubella (Perry et al., 1993; Warrener and Samuel, 2006). The IgG EIA results indicated that oral fluids were a good surrogate for serum and yielded 94% sensitivity and specificity relative to serum specimens in the indirect EIA (Thieme et al., 1994).

Nucleic Acid Detection Techniques

A variety of molecular approaches have been developed for confirmation of detection of mumps infections. Mumps RNA has been detected by RT-PCR in oral fluid, CSF, saliva/throat, and urine specimens (Kashiwagi et al., 1997; Poggio et al., 2000). A TaqMan-based one-step real-time RT-PCR that uses oligonucleotides targeting the matrix protein gene was shown to accurately detect and quantify mumps virus strains (Kubar et al., 2004). Nested RT-PCR, targeting a short fragment of the SH gene, was successfully applied directly to CSF and for identification of mumps virus isolates from cell culture (Palacios et al., 2005). A loop-mediated isothermal amplification method also has been used to amplify and detect mumps virus RNA in clinical samples (Fujino et al., 2005). This method detected slightly more mumps virus-positive samples than cell culture and could detect 0.1 PFU of mumps virus in serial dilution studies (Okafuji et al., 2005). Most recently, real-time RT-PCR assays have been developed for the detection of mumps RNA in clinical specimens using F gene targets (Uchida et al., 2005) and SH gene targets (Krause et al., 2006; Boddicker et al., 2007). These assays are reported to be capable of detecting between 10 and 100 copies of mumps RNA in most specimens tested. The assay designed by Boddicker et al. has been adapted to several different real-time platforms with relative ease, is very well characterized, and is currently in use in a number of public health laboratories in the United States.

Other Methods

Another test for immune status (IgG) is the dot immunobinding assay (Condorelli and Ziegler, 1993). Filter paper soaked in a dilution of patient's serum is applied to mumps antigen dotted onto nitrocellulose. Following 30 min of incubation, the filter paper is removed and the nitrocellulose sheet is washed thoroughly. Next, an enzyme-linked anti-human antibody is added. Finally, the assay is developed with an appropriate chromogen substrate, and the color change is assessed relative to known positive and negative serum specimens. The assay was determined to provide similar results relative to an EIA run in parallel.

Diagnosis in Vaccinated Populations

Confirmation of mumps disease in vaccinated populations has proven challenging. In most instances, the above laboratory methods and procedures perform poorly in previously vaccinated populations. During the recent mumps outbreak in the United States, the majority of patients presenting with symptoms that were clinically compatible with mumps could not be confirmed by a laboratory using serological, virological, or molecular methods that have been so successful in confirming mumps in unvaccinated populations (Dayan et al., 2008).

The serological profile of patients presenting with typical mumps symptoms was IgM negative, IgG positive. IgM-negative EIA serology is not an unusual finding in individuals who receive 1 dose of mumps vaccine and subsequently are either vaccinated a second time or are naturally infected (Sartorius et al., 2005; Krause et al., 2006; Sanz et al., 2006). Difficulties in detection of virus either by cell culture or molecular methods suggests diminished viral loads in previously vaccinated persons. Virus detection was most successful when specimens were obtained between 0 and 3 days after parotitis onset and processed immediately for cell culture or RT-PCR. Even under the most ideal conditions, virus detection by culture and RT-PCR confirmed only 35% of clinically diagnosed mumps cases, whereas mumps IgM was detected in only 13% of patients (CDC, unpublished).

Persons with documented mumps infections can be reinfected with mumps virus later in life (Gut et al., 1995, Crowley and Afzal, 2002; Okafuji et al., 2005; Yoshida et al., 2008). Gut et al. recognized that mumps reinfections could not be reliably confirmed using IgM or IgA serology and noted that IgG avidity and IgG subclass-specific responses

could detect reinfections (Gut et al., 1995). These methods and others which appear capable of detecting avidity differences (Sanz-Moreno et al., 2005) and mumps RNA at very low copy numbers in clinical specimens from vaccinated, reinfected individuals are currently being assessed (Sanz et al., 2006; Yoshida et al., 2008).

At the present time, the recommended tests for laboratory confirmation of mumps disease include detection of IgM in serum and virus detection by culture and real-time RT-PCR. The latter tests should use the contents of a throat swab (Stensen's duct swab) obtained after massaging the cheeks near the parotids or salivary glands for 1 min prior to collection of the specimen. Specimens should be processed for cell culture and molecular methods as soon as possible or kept cold if immediate processing is not possible.

Control and Prevention

In the United States, the mumps vaccine was introduced in 1967 and recommended for routine use in 1977. Between 1968 and 1995, the number of mumps cases decreased from 185,691 to 906, and it decreased to 274 cases in 2001. The enforcement of state vaccination laws requiring students to be vaccinated before school entry has been a major factor in reducing the incidence of the disease (Chaiken et al., 1987). Mumps vaccine is given along with measles and rubella vaccines at 12 to 15 months of age and again at school entry (CDC, 1989).

Despite the routine vaccination of children with mumps vaccine, outbreaks have occurred among older children and adults. Although outbreaks in the 1980s were generally attributed to failure to vaccinate all susceptible children, adolescents, and young adults, more recent outbreaks have occurred among highly vaccinated populations (Hersh et al., 1991; Briss et al., 1994). In 1991, a mumps outbreak was sustained in a population where 98% of individuals had been vaccinated with at least 1 dose of MMR (Hersh et al., 1991). Between December 1997 and May 1998, a mumps outbreak occurred in New York City. Among the 111 cases with known vaccination history, 92% had received at least 1 dose of mumps-containing vaccine, and 62% had received 2 or more doses (Whitman, 1999).

In 2006, the United States experienced the largest mumps outbreak in almost 20 years, largely affecting Midwestern states. Over 6,500 mumps cases were reported, and the highest incidence occurred among college-aged individuals between the ages of 18 and 24 years, 84% of whom had received 2 doses of MMR vaccine (CDC, 2006a, 2006b, 2006c; Dayan et al., 2008). This outbreak has been attributed to secondary vaccine failure and has reaffirmed questions regarding the effectiveness of the currently used mumps vaccines (Harling et al., 2005) and the possibility of waning vaccine-induced immunity. A more controversial reason for the outbreak is the suggestion that mumps vaccine elicits only a partially protective immune response against currently circulating wild-type mumps strains (Nojd et al., 2001; Rubin et al., 2006). Regardless of the reason(s) for such outbreaks, they are disconcerting and suggest that more efficacious vaccines might be required for control and elimination of mumps (Kyaw et al., 2007).

Mumps vaccine is routinely used in only 58% of countries or areas in the world, and importations of mumps into the United States are now increasingly recognized (Galazka et al., 1999). In some European countries, the Rubini mumps vaccine continues to be used, despite its low efficacy. Mumps vaccine has not been found to be effective in preventing infection if given after exposure. Mumps immune globulin is of no value and it is no longer available in the United States.

RUBELLA

Background

Rubella (German measles or 3-day measles) was first described by German authors in the 18th century and was accepted by an International Congress of Medicine in London as a disease independent of measles and scarlet fever in 1881 (Cooper, 1985). It is usually a mild contagious viral disease causing a fine maculopapular rash, which begins on the face and spreads to the abdomen and the extremities. Rubella would be of little medical importance were it not for the profound defects rubella virus (RV) infection can cause in the unborn child. More than 85% of infants born to mothers infected with RV during the first trimester of pregnancy are affected (CDC, 2002). The spectrum of defects includes sensorineural hearing loss, cardiovascular abnormalities, cataracts, and neurologic abnormalities and is collectively known as congenital rubella syndrome (CRS). The association between rubella and birth defects was first recognized by N. McAlister Gregg in 1941, 60 years after the recognition of rubella as a distinct disease (Gregg, 1941). Gregg's insightful discovery occurred when all birth defects were thought to be inherited and rubella was thought to be nothing more than a mild early childhood disease. Since RV is one of the most potent infectious teratogens known, the 60 years between the recognition of rubella and the recognition of CRS illustrates difficulties in recognizing the significance of CRS which persist to the present (Webster, 1998).

Prior to the availability of vaccines against rubella, epidemics occurred every 6 to 9 years in the United States, and larger epidemics occurred at intervals of up to 30 years (Horstmann, 1991). The mechanism(s) by which the major, infrequent epidemics of rubella gain force is not clear, since the virus is transmitted efficiently in families and only one serotype exists (Katow et al., 1997). The last major epidemic in the United States was in 1964 to 1965, resulting in about 11,000 fetal deaths, 2,000 neonatal deaths, and 20,000 children born with CRS (Orenstein et al., 1984; Reef and Cochi, 2006a).

The virus was difficult to detect in tissue culture due to the fact that limited CPE is produced, but it was isolated in 1962 (Parkman, et al., 1962; Weller and Neva, 1962). Clever assays for detection of RV in clinical specimens, based on RV's interference with the replication of lytic enteroviruses, were used (Schiff and Sever, 1966). Attenuation proved to be possible by virus passage in tissue culture, and in 1969, only 7 years after RV was isolated, the first live-virus vaccine was licensed.

Immunization against RV has been consistently recommended in the United States, and indigenous cases of rubella and CRS in the United States have been eliminated, but the worldwide problem is far from over (Reef and Cochi, 2006a). There are more than 100,000 CRS cases each year worldwide (Best et al., 2005).

Description of Virus and Viral Replication

RV is a 60- to 70-nm-diameter particle consisting of a core particle surrounded by a lipid envelope (Banatvala and Best, 1998). The envelope contains two viral glycoproteins, E1 and E2, and the core contains the viral capsid protein (C) and an infectious single-stranded RNA genome of about 9,762 nucleotides. The length of the genome is remarkably

conserved (Zhou et al., 2007). The 5′ 2/3 of the genome codes for a polyprotein which is cleaved into nonstructural proteins necessary for virus replication. Structural proteins (E1, E2, and C) are cleaved from a second polyprotein translated from a subgenomic mRNA produced during viral replication. The subgenomic RNA has the nucleotide sequence of the 3′ 1/3 of the genome. New virions are produced when core particles bud through membranes containing E1 and E2 (reviewed by Chen and Icenogle, 2007). RV is a member of the family *Togaviridae* and is the only member of the *Rubivirus* genus. RV is known to infect only humans, although most other togaviruses are transmitted by insect vectors.

Clinical Aspects

Postnatal Rubella

Postnatal rubella is usually a mild disease requiring little treatment. The virus can be isolated from throat, oral, and nasopharyngeal specimens and, with more difficulty, from blood and urine. Communicability is greatest between about day 14 and day 19 postexposure and is thought to occur from the respiratory tract.

Symptoms of postnatal rubella include a fine maculopapular rash, at about day 14 postexposure, postauricular and suboccipital lymphadenopathy, arthralgia, and low-grade fever at about day 14 postexposure. Postpubertal females often have joint involvement, which may be very painful and may, in rare cases, last a month. The virus has been reported to replicate in synovial membrane cell culture (Grayzel and Beck, 1971; Lund and Chantler, 2000). Rare complications of postnatal rubella include postrubella encephalitis (about 1 in 6,000 cases). Postnatal rubella may be asymptomatic, an important fact when considering potential transmission to the fetus.

Congenital Rubella

The clinical course of rubella in pregnant women is similar to that in nonpregnant women. RV infection, even subclinical infection, can be transmitted to the fetus. The likelihood of defects is highest during the first 11 weeks of gestation. During maternal viremia, about days 7 through 16 postexposure, the placenta may be infected, particularly endothelial cells, and the resulting damage likely allows the virus to cross the placenta (Webster, 1998). In the first trimester, most any fetal organ (heart, brain, and lens cells) can be infected. Fetal spread likely occurs via the vascular system. Spontaneous abortion occurs in about 20% of infections acquired in the first 8 weeks of pregnancy. Neonates with CRS usually have RV-specific IgM but remain virus positive for months. Maternal rubella after the first trimester often leads to infection of fetal tissue, but fetal damage is limited, presumably because organogenesis is complete, transfer of maternal IgG to the fetus is higher, and a fetal immune response is present (Rakowsky and Sever, 1998).

Late manifestations of CRS have been recognized. One of the first disabilities to be found was insulin-dependent diabetes mellitus (Menser et al., 1967; Plotkin and Kaye, 1970). In a follow-up study, 40% of CRS patients from the 1964 rubella epidemic had developed evidence of overt or latent diabetes (Menser et al., 1974). Other endocrine disorders have been seen in small numbers of survivors of congenital rubella. They include hypothyroidism (Ziring et al., 1975; Ziring et al., 1977), hyperthyroidism (Floret et al., 1980), hypoadrenalism (Ziring et al., 1977), and growth hormone deficiency (Preece et al., 1977).

Ocular consequences of CRS are observed during and after the neonatal period. One study described 13 patients having glaucoma 3 to 22 years after birth. Another group of patients were found to have keratic precipitates without other evidence of acute ocular inflammation (Boger, 1980, 1981). CRS has recently been associated with Fuchs heterochromic iridocyclitis (de Groot-Mijnes et al., 2006). A rare disability associated with CRS is progressive rubella panencephalitis (PRP) (Townsend et al., 1976). PRP usually appears during the second decade of life but is very rare (only about 50 cases have been described). Progressive deterioration of intellectual and motor function occurs with dementia close to the time of death. There is an intense immune response against rubella antigens, and high titers of rubella antibody are present in both serum and CSF (Weller et al., 1964). Virus has been recovered from the brain by rescue techniques (Wolinsky, 1978). No correlation has been made between the occurrence of PRP and the presence of rubella-associated defects or the severity of neonatal infection.

The pathology produced with congenital rubella appears to result from a chronic viral infection with alterations of cell multiplication at critical points in organogenesis. This causes the hypoplastic organ development and other characteristic structural defects seen with this disease (Rawls and Melnick, 1966). The immune response may also contribute to permanent damage in the developing child either by an impaired immunity or by inflicting damage through inflammatory mechanisms (Fuccillo et al., 1974; Rosenberg et al., 1981).

Clinical Diagnosis

Postnatal Rubella

Postauricular or suboccipital lymphadenopathy, a fine maculopapular rash, and low-grade fever are characteristic of postnatal rubella, but these are by no means pathognomonic for rubella, since infection with enteroviruses, adenoviruses, measles, and scarlet fever can have similar symptoms. Clinical diagnosis is further complicated because postnatal rubella is a rare illness in some countries, leaving many physicians with little experience with rubella cases. Therefore, laboratory confirmation of RV infection is necessary for an accurate diagnosis.

Congenital Rubella

As with postnatal rubella, the clinical symptoms characteristic of CRS are not unique, and laboratory confirmation of RV infection is necessary. Particular emphasis on laboratory confirmation is necessary in the United States, where CRS is rare.

To identify probable CRS cases and for surveillance purposes, the CDC has adopted the following procedure (CDC, 2007). CRS defects are divided into two groups. Group A consists of cataracts and congenital glaucoma, congenital heart disease (usually patent ductus arteriosus or peripheral pulmonary artery stenosis), loss of hearing, and pigmentary retinopathy. Group B consists of purpura, splenomegaly, jaundice, microcephaly, developmental delay, meningoencephalitis, and radiolucent bone disease. Two symptoms from group A or one from group A and one from group B result in a probable CRS case. The most common defects seen in CRS are hearing loss, cataracts, retinopathy, and congenital heart disease. Laboratory evidence for congenital RV infection confirms a probable CRS case. However, infants may present with a single defect, with hearing impairment being the most common. Therefore, in the United States, the presence of

any defect consistent with CRS and laboratory data consistent with congenital rubella infection results in a CRS diagnosis. Detailed clinical descriptions of CRS are available and are useful in diagnosis (Rakowsky and Sever, 1998). A history of no maternal vaccination and any exposures of a seronegative mother to RV from areas of endemicity during gestation are important pieces of information to gather when determining an index of suspicion for rubella.

Laboratory Diagnosis

Laboratory diagnosis of both postnatal and congenital RV infections is by serologic and/or virus detection techniques. Throat swabs, nasopharyngeal aspirates, or oral fluids are the usual specimens for virus detection. Timing of serum collections is important, particularly for postnatal rubella cases (see below). Antibodies specific for RV antigens can be detected by HI, neutralization tests, EIA, or latex agglutination methods.

A serum positive for RV-specific IgM is diagnostic for recent RV infection. An IgM capture assay is usually preferred because of fewer difficulties, such as false positives, but indirect EIAs that avoid such difficulties by proper absorption steps are acceptable (Tipples et al., 2004). An indirect EIA for RV-specific IgG is usually used (e.g., during prenatal screening for immunity to rubella).

Considerable work on the detection of RV-specific IgM in oral fluids has been done, and this method performs adequately provided a high-quality, sensitive EIA is used (Muller et al., 2007). Very sensitive, low-background methods are necessary when oral fluids are used because antibody levels in this type of specimen are approximately 1/10 the level in sera.

Cell culture-adapted RV strains produce CPE in a variety of cells. Nonadapted viruses from clinical specimens typically do not produce CPE, and additional techniques are required to document the presence of RV in clinical specimens (Chen et al., 2007). Viruses in clinical specimens replicate in a variety of cell lines including Vero, RK13, BHK-21, and GMK-AH-1, and viral replication can be detected by IFA, immunocolorimetric assays, and RT-PCR and by using rubella-derived, self-replicating RNAs expressing green fluorescent protein (GFP) (Zhu et al., 2007) (Color Plate 9). Sensitive standard RT-PCR assays and real-time PCR assays have been developed which allow detection of RV RNA derived from tissue culture and directly in clinical specimens (Jin and Thomas, 2007; Zhu et al., 2007).

Postnatal Rubella

In postnatal infections, IgM is detectable in almost all cases 4 days after disease onset and persists for about 3 weeks (Bellini and Icenogle, 2007). If a serum taken 4 or fewer days after disease onset is negative for RV-specific IgM, testing should be repeated with a serum taken about 5 days later. Alternatively, methods to detect RV RNA may be used. However, the false-negative rates of such tests have typically not been determined. If acute- and convalescent-phase sera are available, a fourfold rise in RV-specific IgG is diagnostic for RV infection. For best results, such sera should be taken within 7 days of rash and 17 to 21 days after rash, respectively. Since virus shedding in throat or nasopharyngeal specimens declines rapidly in the first week after onset of rash, many patients will present when virus shedding is low.

Congenital Rubella

The utility of laboratory techniques is different for CRS than for postnatal rubella. When the risk of postnatal infection is low, the presence of IgM in infants less than 6 months of age is diagnostic of in utero infection. The percentage of cases of congenital rubella infections that are IgM positive declines between 6 and 12 months. Detection of IgM or RV RNA in a newborn with defects consistent with CRS confirms the diagnosis. Virus can be detected in CRS cases for up to 1 year and up to 3 years in some specimens, such as lens aspirates. Before elimination, about half of the CRS cases in the United States were confirmed by RV-specific IgM and half by detection of virus. The presence of maternal RV-specific IgG limits the use of RV-specific IgG in CRS cases. Infant IgG levels which are high or increasing in the first year of life, when maternal IgG declines, are consistent with congenital RV infection. This is particularly useful in areas where postnatal rubella is rare.

Control and Prevention

Rubella is poorly controlled in most of the world. The percentage of seronegative women of childbearing age varies depending on previous epidemics and is sometimes above 25% (Cutts et al., 1997). During rubella outbreaks in unvaccinated populations, lack of immunity in women of childbearing age typically results in 1 to 2 CRS cases per 1,000 births (Cutts et al., 1997).

The means to control rubella is immunization. Safe, effective attenuated live-virus vaccines have been developed by serial passage in tissue culture. The virus used to produce the RA27/3 vaccine, which is used in the United States, as a component of the MMR vaccine, was passaged between 25 and 33 times in WI-38 cells (Reef and Plotkin, 2007). It produces a more wild-type immune response than the previously used vaccine, HPV77DE5, and induces 95% seroconversions of susceptible individuals. It produces the symptoms of mild rubella in 10 to 15% of vaccinees, occasionally even producing a rash. Although virus is shed from the upper respiratory tract, transmission of vaccine virus is very rare. Joint symptoms occur in about 14% of postpubertal women, and rarely, these develop into arthritis. Although pregnancy is a contraindication for vaccination, hundreds of seronegative women have been vaccinated during the first trimester and no fetal defects have been observed (CDC, 2001; Badilla et al., 2007). Control of rubella through vaccination has resulted in elimination of rubella and CRS from the United States. Since 2001, there have been fewer than 25 rubella cases reported annually and only an occasional CRS case (e.g., 1 case in 2004). Since 2001, cases which have been adequately investigated are known to be the result of imported rubella. The elimination of rubella from the United States, a significant public health achievement, was recognized by an independent panel of experts, and the data supporting this conclusion have been summarized in a supplement to *Clinical Infectious Diseases* (Reef and Cochi, 2006b). Since 2003, two WHO regions, the Pan American Health Organization and the European Region, have established goals for rubella and CRS elimination by 2010 and for rubella elimination and prevention of congenital rubella infection by 2010, respectively. In addition, the Eastern Mediterranean Region has established the goal of elimination of CRS by 2010 in those countries that have introduced rubella vaccine into their routine Expanded Program for Immunizations schedule. Countries in other WHO regions are at various stages of introduction of rubella-containing vaccine and establishment of laboratory-based surveillance (Castillo-Solórzano and Andrus, 2004; CDC, 2005).

The first dose of rubella vaccine should be given at 12 to 15 months of age combined with measles and mumps vaccines

(MMR). The second dose of the MMR vaccine should be given at 4 to 6 years of age (CDC, 2008a). Prenatal testing for immunity to rubella and counseling of seronegative pregnant women to avoid contact with rubella cases is an important part of CRS prevention. Prenatal serologic screening of women is indicated for all women who do not have acceptable evidence of immunity based on documented administration of rubella vaccines or laboratory evidence of immunity. Pregnant women who do not have serologic evidence of rubella immunity or documented rubella vaccination should be vaccinated with rubella or MMR vaccine after completion or end of the pregnancy. Women should be counseled to avoid conception for 1 month after vaccination.

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The Human Retroviruses Human Immunodeficiency Virus and Human T-Lymphotropic Virus

JÖRG SCHÜPBACH

32

Retroviruses were for many decades well-known causative agents of leukemias, lymphomas, other cancers, or chronic inflammations in various animal species. The discovery of the first human retrovirus, human T-cell leukemia virus (now renamed human T-lymphotropic retrovirus type 1 [HTLV-1]) was reported in 1980 (Poiesz et al., 1981). HTLV-1 was soon identified as the causative agent of adult T-cell leukemia or lymphoma (ATLL), a rapidly progressing cancer of $CD4^+$ T lymphocytes first described in southeastern Japan (Takatsuki et al., 1977). Knowledge gained from HTLV-1 research was important for the subsequent detection of other human retroviruses, first the related HTLV-2 (Kalyanaraman et al., 1982). Soon thereafter, human immunodeficiency virus type 1 (HIV-1) was, for the first time, isolated from a patient with an early stage of the newly recognized AIDS (Barre-Sinoussi et al., 1983). Two years later, a second AIDS-causing virus, HIV-2, was discovered (Clavel et al., 1986a).

Investigations among nonhuman primates showed a wide distribution of viruses resembling both the HTLV and HIV groups of retroviruses. Simian T-lymphotropic retrovirus type 1 (STLV-1) and STLV-2, simian counterparts of HTLV-1 and HTLV-2, were identified. STLV-3 forms a third group of lymphotropic viruses infecting various African monkey species. HTLV-3, a counterpart of STLV-3 in humans, was recently detected in African pygmies (Calattini et al., 2005; Wolfe et al., 2005; Calattini et al., 2006; Switzer et al., 2006). A further HTLV forming a fourth group, HTLV-4, has also been reported (Wolfe et al., 2005). Together, these viruses now constitute four groups of primate T-lymphotropic retroviruses (PTLV-1, -2, -3, and -4), with representatives in both simians (STLV) and humans (HTLV). Similarly, both HIV-1 and HIV-2 were shown to originate from primate lentiviruses collectively named simian immunodeficiency viruses (SIV).

Other reports of retrovirus infections in humans include isolated cases in which foamy retroviruses (Switzer et al., 2004), simian type-D retrovirus (Lerche et al., 2001), or SIV (Khabbaz et al., 1994) were found in humans as a result of direct cross-species nosocomial transmission from monkeys to caretakers. Transmission of such agents through close and repeated exposure to wild monkeys, for example, in bushmeat hunters, has also been reported (Wolfe et al., 2004). Transmission of animal retroviruses to humans may not be restricted to viruses of primate origin. Of interest are the recent identification of a betaretrovirus closely related to mouse mammary tumor virus in patients with the autoimmune disease primary biliary cirrhosis (Mason et al., 2004; Xu et al., 2004) and the isolation of an infectious xenotropic murine retrovirus in a form of familial prostate cancer characterized by homozygosity for a reduced-activity variant of the antiviral enzyme RNase L (Dong et al., 2007; Fan, 2007). On the other hand, an earlier claim of a novel human retrovirus has received no follow-up confirmation. This relates to the "human retrovirus 5," which now has been identified as a rabbit endogenous retrovirus contaminant, RERV-H (Griffiths et al., 2002). An overview of the currently known exogenous human retroviruses and the diseases associated with them is shown in Table 1. In addition, the question of whether endogenous human retroviruses might contribute to human autoimmune disease like multiple sclerosis, Sjögren's syndrome, systemic lupus erythematosus, and others remains unresolved (Perron et al., 2005; Sander et al., 2005).

SAFETY PRECAUTIONS, DISINFECTION, INJURIES, AND POSTEXPOSURE PROPHYLAXIS

For handling of clinical specimens, all retroviruses, including HIV and HTLV, are classified as biological agents of moderate risk (biosafety level 2). Biosafety level 3 is required for all activities involving propagation of infectious virus. Since the physical compositions of the two viruses are similar, the following information derived from investigation of HIV-1 can be largely applied to the HTLVs.

The risk of laboratory-acquired infection with these viruses stems primarily from contamination of the hands and mucous membranes of the eyes, nose, and mouth by infectious blood and other body fluids. There is no evidence that HIV or HTLV are transmitted by the airborne route. Strict adhesion to the safety precautions is paramount in preventing nosocomial infections (Anonymous, 1991; Collins et al., 1991; Sewell, 1995). Good quality gloves and a protective laboratory gown should always be worn, and eyes should be protected from spills. Disposable unbreakable plasticware should be used, never glassware or other sharp or breakable objects.

TABLE 1 Overview of retroviruses isolated from humans

Virus(es)	Affiliation	Disease associations
HIV-1 and -2	Genus *Lentivirus*; primate lentiviruses	AIDS and related conditions
HTLV-1	Genus *Deltaretrovirus*; PTLVs	ATLL, HAM/TSP, other HTLV-1-associated inflammatory disorders
HTLV-2	Genus *Deltaretrovirus*; PTLVs	Low pathogenicity; cases of HAM/TSP and other neurological disorders; inflammatory disorders
HTLV-3	Genus *Deltaretrovirus*; PTLVs	Unknown
HTLV-4	Genus *Deltaretrovirus*; PTLVs	Unknown
Human foamy virus	Genus *Spumavirus*	Nosocomial infection with no known disease association
SIV	Genus *Lentivirus*; primate lentiviruses	Nosocomial infection with too short observation
Simian type-D retrovirus	Genus *Deltaretrovirus*	Nosocomial infection with too short observation
Mouse mammary tumor virus-like	Genus *Betaretrovirus*	Primary biliary cirrhosis?
Xenotropic murine retrovirus	Genus *Gammaretrovirus*	Familial prostate cancer associated with reduced RNase L activity?

Spills or contaminations of laboratory surfaces must be decontaminated immediately. Whenever possible, a type 2 laminar flow biological safety cabinet should be used when handling patient samples. Centrifuges, including those of laboratories that perform only serology, should be equipped with sealed buckets. HIV, HTLV, and other retroviruses are rapidly inactivated by detergents and disinfectants that are effective against enveloped viruses. Otherwise, at least HIV is relatively stable. At autopsy, HIV was isolated up to 16.5 days postmortem from various tissues (Douceron et al., 1993). Suspensions of the virus in protein-containing fluids or dried preparations are also relatively stable (Tjotta et al., 1991). At the optimum pH of 7.1, the half-life ranged from about 24 h at 37°C to no significant loss over 6 months at –75°C. Drying the virus on a glass surface and freezing caused 5- to 12-fold and 4- to 5-fold decreases in activity, respectively. The dried preparations, however, were about as stable as when stored in a buffered solution (Tjotta et al., 1991). In another study, 1 $\log_{10}$ of inactivation in culture fluid, seawater, sewage, and dechlorinated tap water (all sterile and kept at 16°C in the dark) required 1.3, 1.6, 2.9, and 1.8 days, respectively. After the first 4 days, the inactivation became even slower (1 $\log_{10}$ inactivation after 4.3, 2.6, 5.7, and 4.6 days, respectively). HIV was more stable than herpes simplex virus but less stable than poliovirus (Sattar and Springthorpe, 1991). These data are not meant to suggest that HIV transmission might occur by exposure to water, for which there is absolutely no basis. They should, however, make clear that caution is important when working with HIV.

The standard disinfectant recommended for contaminated surfaces is a hypochlorite solution with a concentration of 0.5% available chlorine (5 g/liter, 5,000 ppm). When working with HIV cultures and virus preparations, a higher concentration of 1% available chlorine is recommended (Anonymous, 1991; Van Bueren et al., 1995). Fresh 2% solutions of alkaline glutaraldehyde are effective, but care should be taken that they are not too dilute or have not become stale when used for disinfecting HIV associated with organic matter. A solution of iodine and detergent (2% Jodopax) will remove all detectable HIV-1 activity. In contrast, 70% industrial methylated spirit or 70% ethanol is not effective in inactivating dried protein-rich spills of cell-free or cell-associated HIV within a reasonable amount of time; complete inactivation requires up to 20 min (Tjotta et al., 1991; van Bueren et al., 1994).

The risk of HIV infection following percutaneous needlestick exposure to HIV-contaminated blood is estimated to be between 0.13 and 0.5%. It depends on the depth of the penetration (relative risk [RR] of percutaneous lesions, 16.1), visible contamination of the penetrating object with blood (RR, 5.2), prior use for an intravenous or intra-arterial injection (RR, 5.1), and disease stage (with respect to viral load) of the index patient (RR, 6.4) (Centers for Disease Control and Prevention, 1995). Needlestick or other puncture wounds, cuts, and skin contaminated by spills or splashes of specimen material should be thoroughly washed with soap and water and disinfected with a nonirritating disinfectant. Bleeding should be encouraged. In case of percutaneous injury or contact of mucous membranes or nonintact skin (e.g., exposed skin that is chapped, abraded, or afflicted with dermatitis) with blood, tissue, or other body fluids potentially infectious, an antiretroviral postexposure prophylaxis should be started immediately according to guidelines published online (http://www.hivatis.org/Guidelines/Default.aspx?MenuItem=Guidelines). Note that these recommendations do not apply to HTLV, as many of the drugs effective against HIV, particularly the protease inhibitors and nonnucleoside reverse transcriptase inhibitors, are ineffective against HTLV.

HIVs

Biology and Epidemiology

HIV-1 and HIV-2 are members of the genus *Lentivirus* of the *Retroviridae* family. They are enveloped plus-strand RNA viruses, with a diameter of about 110 nm. Infectious particles (virions) contain two identical copies of single-stranded RNA of about 9 to 10 kb. These are surrounded by structural proteins that form the nucleocapsid and the matrix shell, surrounded by a lipid envelope derived from the host cell membrane. Viral glycoprotein trimers which mediate adsorption to and penetration of the host cell membrane are inserted in this envelope (Fig. 1).

HIV-1, first isolated in 1983 (Barre-Sinoussi et al., 1983) and confirmed in the following year as being virologically

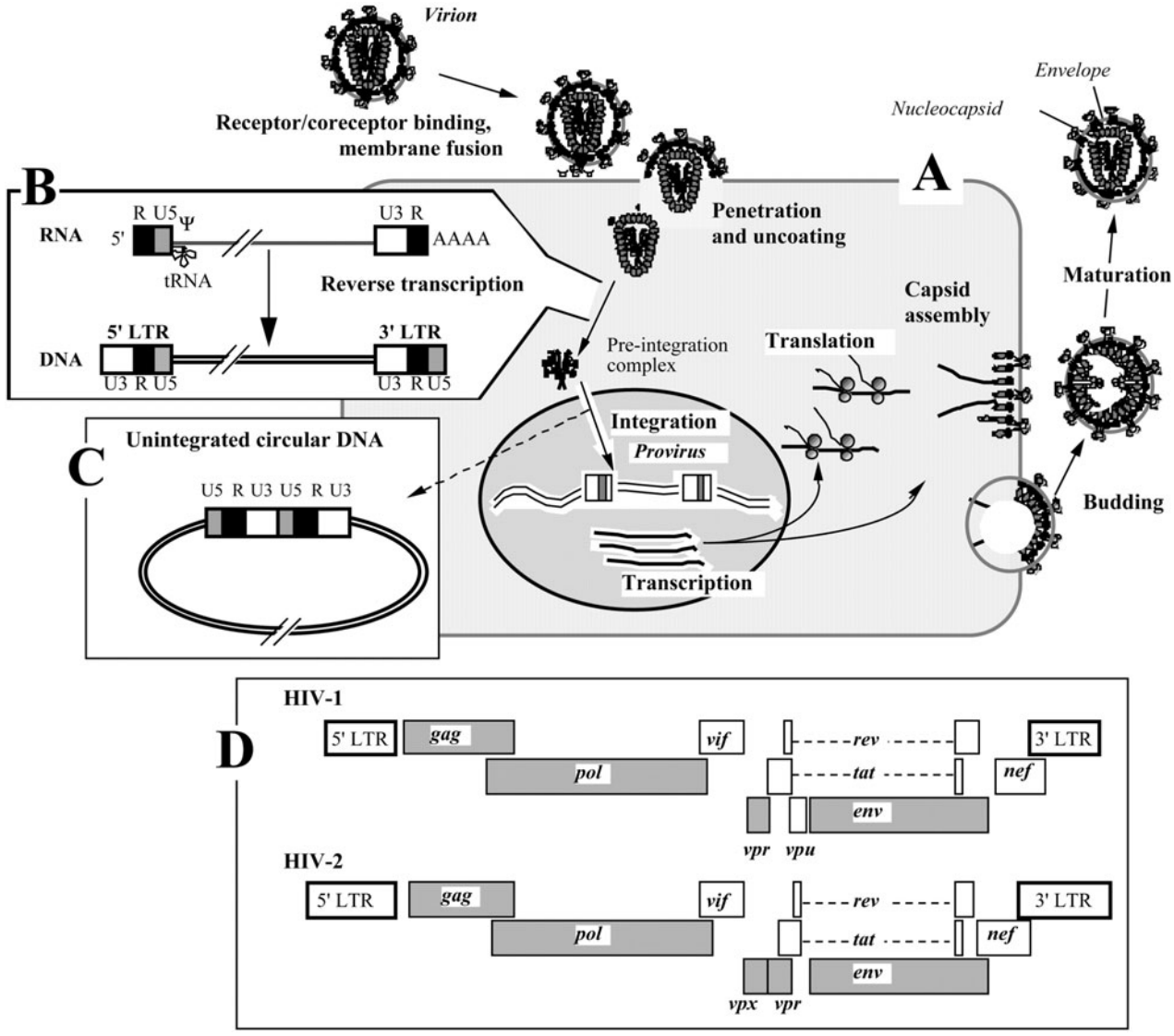


FIGURE 1 HIV replication cycle. (A) Overview. (B) Reverse transcription. The retroviral genome contained in virions consists of RNA. Its characteristic features include terminal repeats (R), U5 (5′ untranslated), U3 (3′ untranslated), 3′ polyadenylation, a binding site for a tRNA which serves as the primer for reverse transcription, and the encapsidation signal Ψ. During reverse transcription, the viral RNA is reverse transcribed into double-stranded DNA, and terminal sequences are partially duplicated in a way that leads to an LTR composed of U3-R-U5. (C) Unintegrated circular DNA is a short-lived by-product of provirus integration; its presence in a cell sample indicates actively replicating virus. (D) Genomic organization of HIV-1 and HIV-2. The hatched boxes denote ORFs for proteins which are contained in particles. Drawing modified from Schüpbach, 2003b.

and serologically associated with early and late stages of AIDS (Gallo et al., 1984; Levy et al., 1984; Popovic et al., 1984; Sarngadharan et al., 1984; Schupbach et al., 1984), is the more aggressive virus and responsible for the AIDS pandemic. HIV-2, discovered in 1986 (Clavel et al., 1986b), is less pathogenic. Rates of heterosexual and mother-to-child transmission of HIV-2 are low, and latency dominates the clinical picture; the virus rarely causes AIDS (Schim van der Loeff and Aaby, 1999; Bock and Markovitz, 2001; Jaffar et al., 2004).

Origin of HIV

A group of related viruses, SIV, naturally infect various species of Old World monkeys and the chimpanzee (Fig. 2A). These primate lentiviruses are categorized into five major lineages. Lineage 1 contains the various isolates of HIV-1, which are subclassified into three groups, M (main), O (outlier), and N (Simon et al., 1998). From the phylogenetic tree it is evident that group M isolates (e.g., HIV-1/LAI) are more closely related to two isolates from chimpanzee, SIVcpzGAB1 and SIVcpzUS, than to isolates of HIV-1 group O (HIV-1/ANT70) or to another chimpanzee isolate, SIVcpzANT. These data indicate that the HIV-1 epidemic is the result of zoonotic virus transmissions from the chimpanzee, subspecies *Pan troglodytes troglodytes*, to humans (Gao et al., 1999). The origin of group M diversification, i.e., the beginning of the HIV-1 pandemic, is placed around

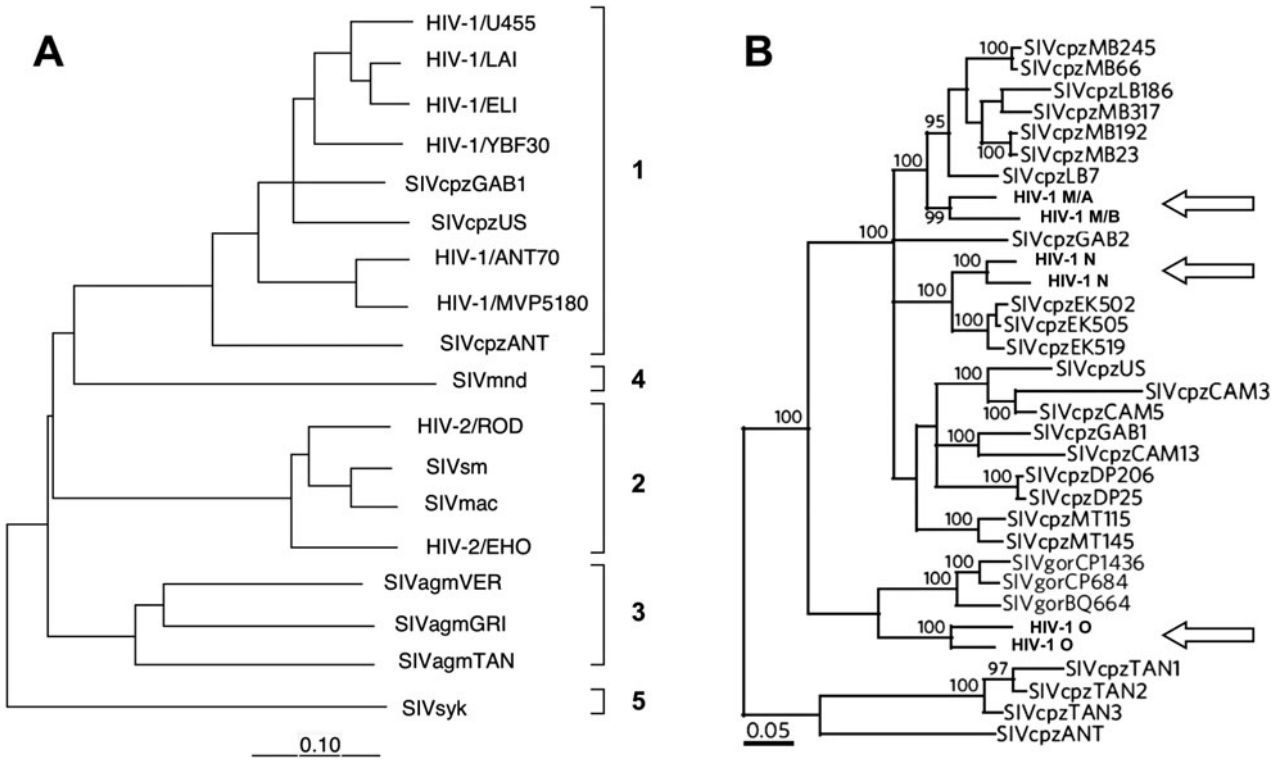


FIGURE 2 Origin of HIV-1 and HIV-2. (A) Phylogenetic tree of primate lentiviruses, derived from Pol protein sequences. Numbers 1 to 5 indicate the five major lineages. HIV-1/U455 is a group M, subtype A isolate. ELI is of group M, subtype D, and LAI is of group M, subtype B. ANT70 and MVP5180 represent group O, and YBF30 is group N. ROD and EHO represent different subtypes of HIV-2. SIVcpzGAB1, SIVcpzUS, and SIVcpzANT are chimpanzee (cpz) isolates. mnd, mandrill; agm, African green monkey; syk, Sykes' monkey; sm, sooty mangabey. The bar at the bottom denotes genomic diversity. (B) Phylogenetic tree showing the relationship of HIV-1 groups M, N, and O to chimpanzee and gorilla (gor) lentiviruses, respectively. Combined and modified from Sharp et al., 1994, Gao et al., 1999, and Van Heuverswyn et al., 2006.

1930 (Korber et al., 2000; Salemi et al., 2001). Recent investigations involving HIV serology and reverse transcriptase PCR (RT-PCR) performed on fecal samples collected in big ape habitats in Cameroon have demonstrated a wide variety of SIVcpz isolates, which are organized in phylogenetic clades restricted to the respective habitat area. SIVcpz prevalence in some habitat areas is as high as 23 to 35%, while in others it is only a few percent or absent. Phylogenetic analysis of SIVcpz together with HIV-1 isolates clearly shows that HIV-1 group M originates from SIVcpz isolates that are prevalent in two *P. troglodytes troglodytes* populations living in the extreme southeast of Cameroon. Moreover, HIV-1 group N originated from SIVcpz isolates from *P. troglodytes troglodytes* living in a different area located about 250 km to the west-northwest (Keele et al., 2006). Wild chimpanzees therefore act as a reservoir for HIV-1 groups M and N. Viruses closely related to HIV-1 group O have been isolated from gorillas living in forest habitats of Cameroon 400 km apart from each other (Van Heuverswyn et al., 2006). Phylogenetic analysis demonstrates that both HIV-1 group O and SIVgor have originated from chimpanzee viruses (Fig. 2B). Whether chimpanzees transmitted HIV-1 group O viruses to gorillas and humans independently, or to gorillas that then transmitted it to humans secondarily is unknown.

Lineage 2 of primate lentiviruses contains the various isolates of HIV-2, which are related to viruses infecting sooty mangabeys (SIVsm). SIVsm also has been transmitted naturally to macaques. HIV-2 strain ROD differs less from SIVsm or SIVmac than it does from another human isolate, HIV-2/EHO (Fig. 2A). This, together with other similar examples, has led to the conclusion that the HIV-2 epidemic is also the result of multiple simian-to-human cross-species transmissions. Transmission of the epidemic subtypes HIV-2A and B may have occurred around 1940 (Lemey et al., 2003).

HIV Groups and Subtypes

The extraordinary variability of HIV, due to rapid mutation and recombination, has led to the development and geographical distribution of various distinctive clades, or subtypes, of viruses (McCutchan, 2000; Peeters and Sharp, 2000). HIV-1 group M is divided into subtypes A, B, C, D, F, G, H, J, and K. Genetic variation within a subtype can be on the order of 15 to 20%, whereas variation between subtypes is approximately 25 to 35%, depending on the subtypes and genome regions examined (Korber et al., 2001). Viral recombination, a consequence of infection in a person by more than one virus (coinfection or superinfection), has furthermore resulted in a great variety of so-called circulating recombinant forms (CRFs), which increasingly dominate the epidemic. To date, more than 20 CRFs have been defined, based on their identification in at least three epidemiologically unlinked individuals and characterization of the full-length sequence. According to a WHO study involving

23,874 HIV-1 samples from 70 countries, subtype C accounted for 50% of all infections worldwide in 2004. Subtypes A, B, D, and G accounted for 12, 10, 3, and 6%, respectively. Subtypes F, H, J, and K together accounted for 1%. The circulating recombinant forms CRF01_AE and CRF02_AG each were responsible for 5%, and CRF03_AB was responsible for 0.1%. Other recombinants accounted for the remaining 8% of infections. All recombinant forms together were responsible for 18% of infections (Hemelaar et al., 2006). Isolates of group O, which are almost exclusively restricted to persons originating from Cameroon, Gabon, and Equatorial Guinea, differ as much from each other as do viruses from different subtypes of group M, but their limited number has so far precluded a definition of distinct subtypes. Group N viruses were isolated from only a few individuals from Cameroon (Simon et al., 1998). A total of seven subtypes of HIV-2, two of which are epidemic (A and B) and five of which are nonepidemic (C to G), have been defined, resulting from the same number of different simian-to-human transmissions (Lemey et al., 2003).

Of all HIV-1 infections worldwide, 64% are present in sub-Saharan Africa. In 2004, 56% of infections in that region were caused by subtype C, with smaller proportions caused by subtype A (14%) or G (10%), CRF02_AG (7%), and other recombinants (9%). Subtype C accounts for more than 97% of the infections in Southern Africa, Ethiopia, and India and for significant proportions of infections in East, North, and Central Africa. Subtype A is responsible for one-third of the infections in East and Central Africa, one-fifth in West Africa, and 80% in Eastern Europe and Central Asia. Subtype B, until 2 decades ago solely responsible for the epidemic in North America, the Caribbean, Latin America, Europe, and Australia, now has a share of 75 to 95% in these regions. Subtype D accounts for 10 to 15% of infections in Central and East Africa and about half of those in North Africa. Subtype G accounts for one-third of infections in West Africa and above 10% in Central Africa. Subtypes F, H, J, and K have remained minority populations in all world regions (Hemelaar et al., 2006). In contrast, the recombinant forms are of increasing relevance. CRF01_AE and CRF02_AG are causing heterosexual epidemics in Asia and West Africa, respectively. CRF01_AE is responsible for 85% of the infections in South and Southeast Asia and 16% in East Asia. CRF02_AG accounts for one-third of new infections in West Africa and about 6.7% in Central Africa (Njai et al., 2006). Non-B subtypes account for an increasing proportion of newly diagnosed HIV-1 infections in Europe (Böni et al., 1999; Lot et al., 2004).

The HIV Replication Cycle

An overview of HIV replication is given in Fig. 1A. Like all retroviruses, HIV particles contain a characteristic enzyme, RT. The enzyme is cleaved, and thereby activated, from a precursor protein by the action of another retroviral enzyme, the viral protease (PR). RT possesses three distinct enzymatic functions. It acts as an RNA-dependent DNA polymerase (the RT activity in the strict sense of the word), an RNase H, and a DNA-dependent DNA polymerase. After infection of a host cell, these different RT functions serve in turn to synthesize a cDNA of the viral RNA, to degrade RNA from the cDNA-RNA heteroduplex, and to duplicate the cDNA strand. Regulatory sequences present at both ends of the viral RNA (R-U5 at the 5′ end and U3-R at the 3′ end) are thereby complemented and partially duplicated in a manner that yields the long terminal repeats (LTR). These contain U3-R-U5 and are located at both ends of the double-stranded viral DNA (Fig. 1B). This double-stranded DNA, associated with the proteins of the preintegration complex, migrates into the nucleus, where it is integrated into the host cell genome by a third retroviral enzyme, the integrase (IN). The integrated retroviral DNA genome is called the provirus. A short-lived byproduct of replication, unintegrated circular DNA which contains one or two LTRs, has been used as a marker for ongoing viral replication in patients receiving effective long-term antiretroviral combination treatment (Pauza et al., 1994; Furtado et al., 1999) (Fig. 1C).

The genomic organization of HIV-1 and HIV-2 proviruses is shown in Fig. 1D. Like all retroviruses, HIVs possess the open reading frames (ORFs) *gag* and *env*, which code for structural proteins, namely, the precursor proteins of the viral capsid and the envelope, and *pol*, which codes for the enzymes. Additional overlapping ORFs code for the *trans*-acting transcriptional activator (Tat) and the regulator of viral expression (Rev), which are both essential for virus replication. Furthermore, both HIV types contain ORFs for several accessory or auxiliary proteins including Vif, Vpr, Vpu, and Nef (in HIV-1) or Vif, Vpx, Vpr, and Nef (in HIV-2).

While host cell infection and provirus integration are largely mediated by the proteins carried in the virion, the production of viral RNA, structural proteins, and enzymes involves cellular enzymes associated with transcription and translation but also a number of viral regulatory proteins, namely, Tat, Rev, Nef, and Vpr (Stevens et al., 2006; Zhou and Yik, 2006; Balvay et al., 2007). Particles are assembled at the cell membrane and, while still immature and noninfectious, released by budding. For full maturation into infectious particles, the viral Gag and Gag-Pol precursor proteins must be cleaved by PR into the different subunit proteins. An overview of the different viral proteins and their positions in the mature particle is given in Fig. 3. One virion contains 2 copies of genomic RNA, about 1,200 molecules of each Gag protein, 80 molecules of RT, but on average, only about 10 gp120 knobs, corresponding to 30 molecules (Layne et al., 1992).

Virus Entry into Host Cells

For infection of a host cell, the virion must bind via gp120 to a membrane-located virus receptor, which is the CD4 molecule. Each monomer of gp120 contains a binding site for CD4. Some cell types targeted by HIV in vivo express high levels of CD4 (for example, T cells); others, like macrophages and dendritic cells (DC), express very little. In these instances, HIV may initially attach to cells by CD4-independent mechanisms, including interaction of sugar groups on gp120 with other sugars or lectin-like domains on cell surface receptors. Furthermore, cell surface proteins with high affinity to gp120 are expressed on certain DC populations (DC-SIGN) and on endothelial cells (DC-SIGNR). Gp120 also binds the glycolipid galactocerebroside and its sulfated derivative, sulfatide. These molecules are expressed on neurons and glia in the brain, colon epithelial cells, and macrophages. In all instances, interaction of gp120 with CD4 is, however, needed to induce conformational changes in the gp120 trimer that enable interaction with a coreceptor, a molecule of the family of seven-transmembrane chemokine receptors. This interaction is followed by another conformational change of gp120, allowing insertion of the fusion domain of the virion's transmembrane protein, gp41, into the host cell membrane. This leads to fusion of the viral and cellular membranes and viral entry (reviewed by Clapham and McKnight, 2002; Moore et al., 2004).

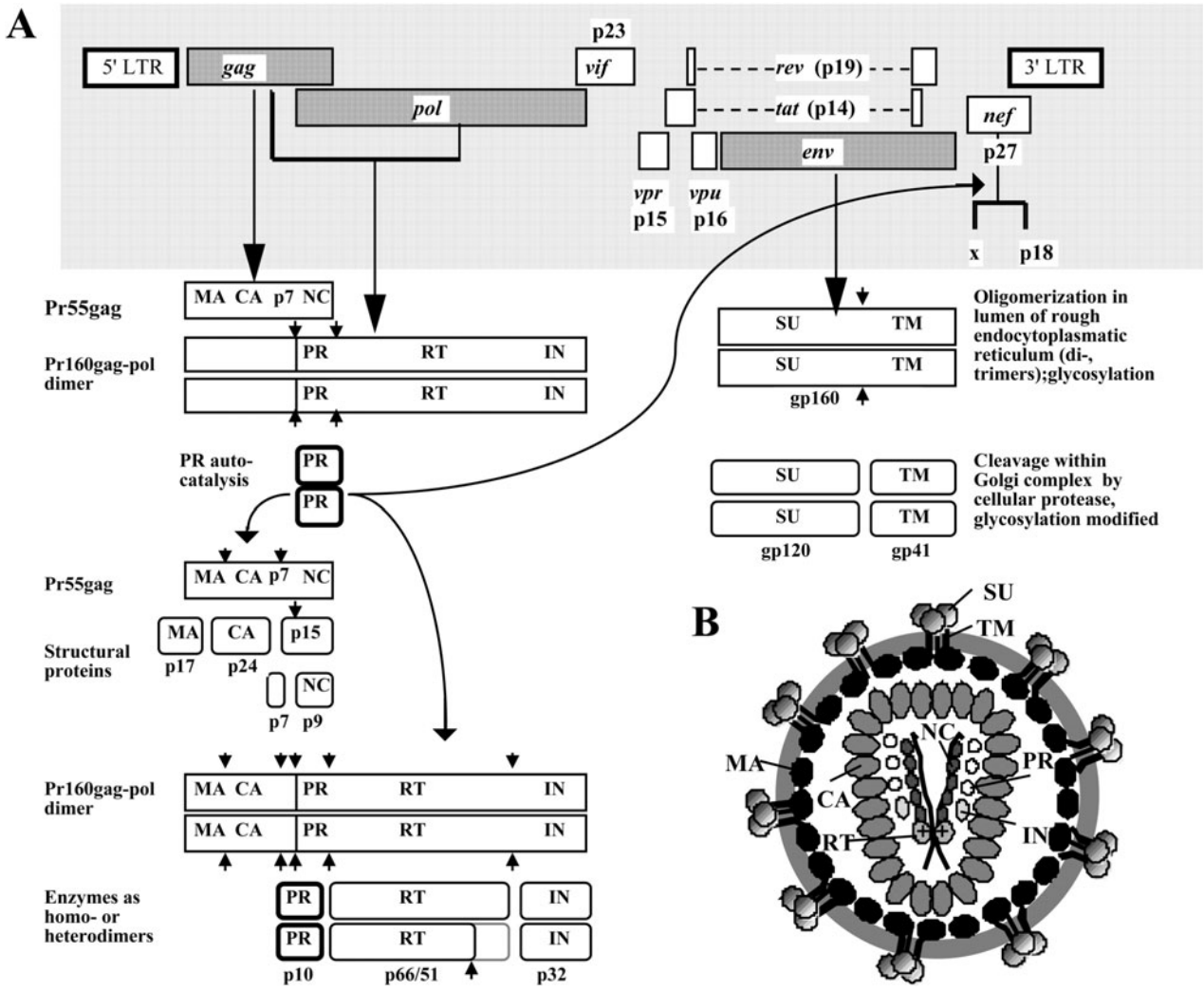


FIGURE 3 Translational products of HIV-1 and particle composition. (A) Translation. The open boxes in the genome representation at the top denote ORFs of the accessory proteins Tat, Rev, Nef, Vif, Vpr, and Vpu, which are translated into proteins of final size. The hatched boxes denote ORFs translated into precursor proteins. The products of the *gag*, *pol*, and *env* genes are synthesized as polyprotein precursors. The principal Gag precursor, $Pr55^{Gag}$, is cleaved by the viral protease (PR or p10) into the matrix (MA) protein p17, the capsid (CA) protein p24, and a C-terminal protein p15, which is subsequently cleaved into p7 and the nucleocapsid (NC) protein p9. Cleavage of $Pr160^{Gag\text{-}Pol}$, which is produced by ribosomal frameshifting at the *gag-pol* junction, yields PR, RT, and IN. All three enzymes remain dimerized after cleavage. RT first forms a homodimer, p66-p66, which is subsequently modified into the heterodimer, p66-p51. The Env precursor gp160 is glycosylated in the Golgi system, oligomerizes into dimers and trimers, and is cleaved by a cellular protease into the SU protein gp120 and the smaller TM protein gp41. The small arrows indicate protease cleavage sites. (B) Localization of viral proteins in mature virions.

The chemokine coreceptors are G-protein-coupled signaling receptors which bind chemokines involved in controlling the activation of various leukocytes and their migration to a site of infection. In vivo, HIV replication is restricted to hematopoietic cells that express CD4 and CCR5 and/or CXCR4. Cells that express CCR5 can be infected by so-called R5 viruses (previously called macrophage-tropic viruses or non-syncytium-inducing viruses). CCR5-mediated HIV infection is inhibited by the natural ligands of CCR5, the beta-chemokines RANTES, MIP-1α, MIP-1β, and monocyte chemoattractant protein 2 (Cocchi et al., 1995) and by a new class of antiretroviral drugs, CCR5 antagonists. The main target cells of R5 viruses in vivo are T lymphocytes of the $CD4^+CD45RO^+$ memory cell phenotype and, to a lesser degree, $CD4^+CD45RA^+$ naive cells. Monocytes, various tissue macrophages, and DC are also infected by R5 viruses (Montaner et al., 2006). Viruses that enter cells via CXCR4 are called X4 isolates (Berger et al., 1998). In contrast to R5 viruses, which only infect primary cultures of lymphocytes or macrophages but no T-cell lines in vitro, X4 viruses also infect T-cell lines and were thus called T-cell tropic, syncytium-inducing viruses. The natural ligand of CXCR4 is the stroma-derived factor SDF-1 (Bleul et al., 1996; Oberlin et al., 1996); new investigational drugs inhibit CXCR4-mediated infection. When X4 viruses emerge in vivo, their tropism is broader and new cell populations are targeted, as CXCR4

expression is more widespread and predominates on naive T cells. Current data support a model where R5 viruses predominate early in the asymptomatic phase, before strains able to use CXCR4 and often several other coreceptors ($R5X4^{++}$ viruses) emerge (Scarlatti et al., 1997).

Aspects of HIV Expression

Host cell activation induces transcription of the viral genes from the promoter located in the U3 region of the 5′ LTR (Fig. 1B). HIV transcription is enhanced by a number of cellular activation factors, and therefore, the virus replicates better in activated cells (Stevens et al., 2006). Virus levels consistently increase when the immune system is activated, for example, by infections or immunogens such as influenza or tetanus toxoid vaccines (Lawn, 2004). Virus production is also enhanced by certain cytokines, namely the proinflammatory cytokines tumor necrosis factor alpha, interleukin-1β (IL-1β), and IL-6 (Hunt, 2007). It has been estimated that the total number of virions that are produced and released in an untreated HIV-1-infected individual is on the order of 10^{10} per day (Simon and Ho, 2003). Conversely, the immune system is activated by HIV expression (Smith, 2006). Inside infected cells, Nef activates signal transduction pathways, namely, the NF-κB system, thereby enhancing viral transcription (DeLuca et al., 1999; Baba, 2006; Stevens et al., 2006). Chronic production of viral antigens activates lymphocytes of corresponding specificity. In addition, the binding of gp120 to CD4 nonspecifically activates $CD4^+$ T lymphocytes (Misse et al., 2005). This permanent stimulation causes a chronic hyperactivation of the immune system, thus constituting a vicious cycle leading to new virus expression and killing of $CD4^+$ T lymphocytes (Fauci, 1993; Lawn et al., 2001). Efficient antiretroviral combination therapy decreases the levels of viral proteins by blocking new host cell infection, thus leading to a near-normal state of immune system activation (Autran et al., 1997). Unfortunately, this also drives the virus into proviral latency, in which it can be attacked neither by the immune system nor the therapy, which is effective only against replicating virus (Marcello, 2006; Stevens et al., 2006).

Sequence Diversity as a Result of RT Errors and Recombination

Retroviral RTs do not possess a proofreading activity and thus have a high misincorporation rate. Additional errors may occur during transcription, since RNA polymerase II does not proofread either. For the 9.5-kb HIV genome, the in vivo error rate is estimated to amount to one to three misincorporations per replication cycle (Coffin, 1992). Given the high rate of virus replication, every single mutation at every possible position of the 9.5-kb-long genome could arise daily. Another mechanism contributing to sequence diversity is genomic recombination, which may occur after coinfection of a cell with two different viruses and encapsidation of both viral RNAs in the same particle (heterozygosity). Its frequency is estimated at 2 to 3 events per viral genome and replication cycle (Jetzt et al., 2000; Zhuang et al., 2002). Recombination is well documented in CRFs of HIV-1, which are evidence of intersubtype recombination (see above). Recombination may have played a key role in the recent evolution of HIV-1, and the geographic intermixing of subtypes, which is increasing, is likely to foster the emergence of an even greater variety of recombinant strains.

Sequence diversity is manifested not only on the level of the pandemic but also in the infected individual in whom it is generated. The rapidity with which virus replicates is an important factor contributing to the accumulation of virus variants. Selective pressure factors, such as the local availability of host cell receptors or coreceptors, cellular or humoral antiviral immune responses, or antiretroviral drugs may then act on this pool of variant viruses, inhibiting the growth of some variants and favoring the replication of others that exhibit a better-suited phenotype. The outgrowth of such a group of viruses under selection pressure is called a quasispecies (Wain-Hobson, 1992). The many quasispecies in each patient evolve both in time and space. It is estimated that the sequence variability in an infected person increases by about 1% per year. In a given patient, different quasispecies are present at different sites in the body, for example, in Langerhans' cells of different skin patches (Sala et al., 1994), individual microdissected splenic white pulps (Cheynier et al., 1994), brain, or genital tract (Zhu et al., 1996).

Virus Transmission and Establishment of Infection

HIV is transmitted predominantly by sexual intercourse, connatally from mother to child, postnatally by breast-feeding, or by parenteral inoculation. Globally, the most frequent route of transmission is by sexual intercourse. The probability of HIV-1 transmission per unprotected coital act is estimated at 1/10 to 1/1,600 for male-to-male transmission, at 1/200 to 1/2,000 for male-to-female transmission, and at 1/200 to 1/10,000 for female-to-male transmission. The average risk is 0.5 to 1% for one-time injecting drug use, 12 to 50% for connatal mother-to-child transmission, 12% for breast-feeding, 90% for a contaminated blood transfusion, and 0.1 to 1.0% for nosocomial transmission (reviewed in Levy, 1997). In general, the risk is proportional to the viral load. The virus is not transmitted through casual contact in household settings, and there is no evidence for transmission by nonhuman vectors.

Sexual transmission is mediated by infectious HIV-1 and/or infected cells in semen or mucosal secretions. The relative transmissibility of cell-free versus cell-associated virus is unknown. The risk of transmitting or acquiring infection varies greatly. Epidemiologic studies indicate that transmission is linked to viral shedding, i.e., the amount of infectious virus in genital fluids. This in turn is linked to the disease stage and is highest during acute infection and late-stage AIDS (see Fig. 5). Effective antiviral therapy can reduce HIV-1 shedding in semen and the female genital tract to undetectable levels, but virions can sometimes be found in semen even when they are undetectable in the blood plasma. Thus, although some untreated infected individuals pose a low transmission risk, others may be "super shedders" and highly infectious. Acutely infected individuals pose a particular risk. Moreover, other sexually transmitted diseases have a marked effect on both viral shedding and the risk of acquiring HIV-1 infection (reviewed in Kaul et al., 2008).

For sexual transmission, virions or infected cells must cross the epithelial barriers of the female or male genital tract (reviewed in Shattock and Moore, 2003; Kaul et al., 2008). The multiple layers of stratified squamous epithelium that line the most exposed regions of the female and male genital mucosa (vagina and ectocervix in women; inner foreskin, penile glans, and fossa navicularis in men) constitute a significant physical barrier. It may be transgressed through physical breaches or by infection of intraepithelial Langerhans cells. The single-layered columnar epithelium which lines the endocervix is more fragile than the stratified epithelium, especially when present as cervical ectopy located on the exocervix and exposed directly to physical

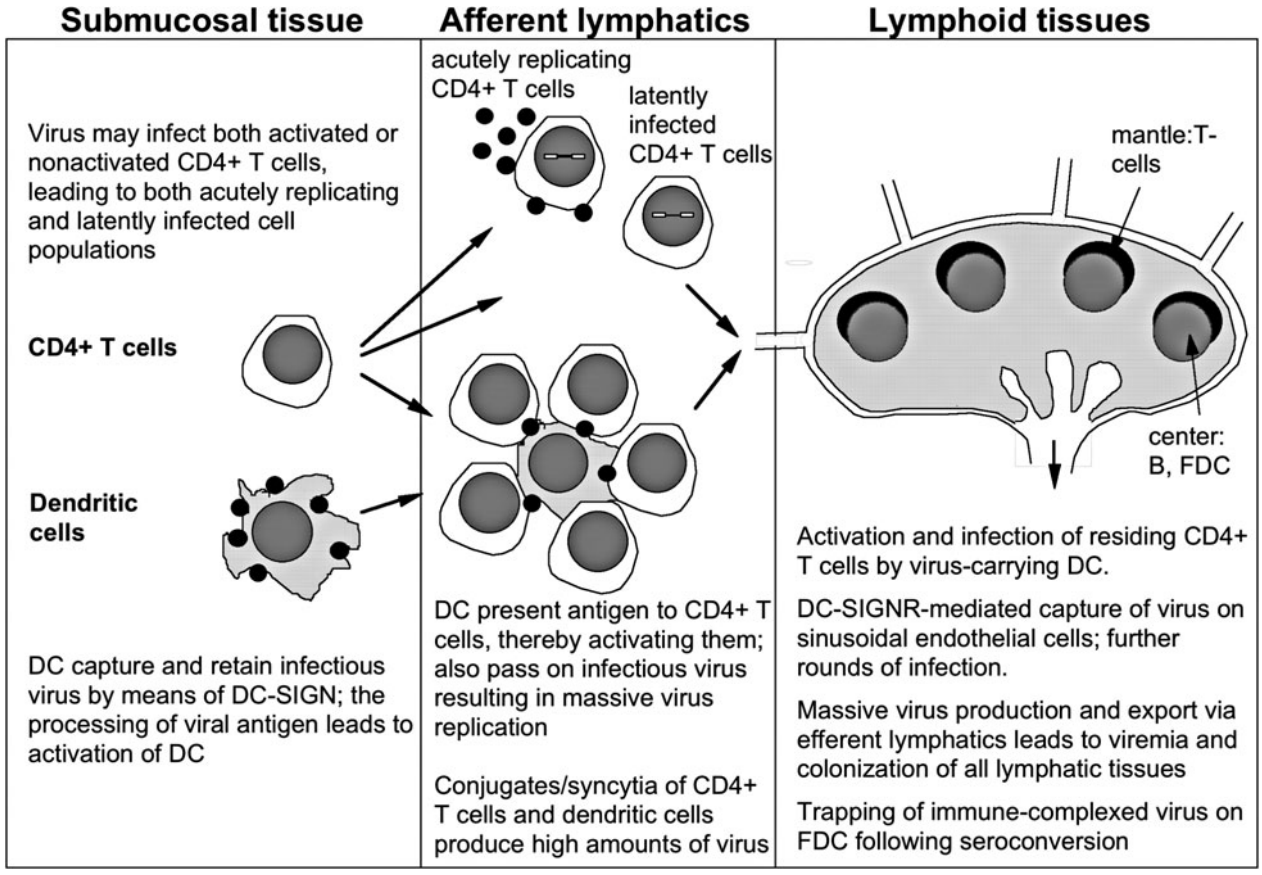


FIGURE 4 Propagation of HIV from the mucosal entry port to the lymphatics and the bloodstream. FDC, follicular DC.

stress. The single-layered rectal epithelium likewise provides little protection against potential trauma during intercourse, facilitating HIV-1 access to the underlying target cells and even the systemic circulation. Moreover, the rectum, unlike the genital tract, is populated with organized lymphoid tissues (lymphoid follicles). The epithelium also contains specialized M cells capable of binding and presenting HIV-1 to the underlying lymphoid tissue. Such physiological and anatomical differences could account for the greatly increased risk of acquiring HIV-1 infection during anal intercourse.

Both the genital and rectal subepithelial stromal tissues are densely populated with DC, macrophages, and T cells that express CD4, CCR5, and to a lesser extent, CXCR4 and are susceptible to HIV-1 infection. Any break in epithelial integrity permits virions direct access to these target cells, allowing the establishment of infection in mucosal sites (Fig. 4). Infection of these cells can be detected within 1 h of the addition of SIV to the macaque vagina and is most commonly observed where the epithelium is abraded (reviewed in Miller and Shattock, 2003).

The peroral route of infection is involved in the many mother-to-child transmissions through breast-feeding, but whether the site of actual virus transmission is within the oral cavity or in the small intestine is unclear (Herzberg et al., 2006). Oral transmission also has been implicated in cases in which the only risk factor was receptive oral intercourse (reviewed by Campo et al., 2006; Syrjanen, 2006). In parenteral infections, the likely primary target cells of intravenously inoculated virus consist of DC, which further transmit the virus to circulating $CD4^+$ T cells (Cameron et al., 2007).

For the sexual transmission of HIV at mucosal surfaces, DC are considered to play an important role (reviewed in Teleshova et al., 2003; Wu and KewalRamani, 2006). DC include Langerhans cells, which are nonmigratory, in epithelial and mucosal tissues, and immature DC of myeloid origin in the submucosa. Upon contact with antigen, the myeloid DC are activated and migrate through the afferent lymphatics to the T-lymphocyte-rich areas of regional lymph nodes, where they present the antigen to T cells. Tissue culture studies have shown that DC can capture and transmit HIV to $CD4^+$ T cells, mainly through DC-SIGN, which interacts with gp120 (Geijtenbeek and van Kooyk, 2003). In vivo, the immature DC with the captured HIV migrate to lymphoid tissues and transmit the virus to activated $CD4^+$ T lymphocytes (Fig. 4).

The availability of densely packed $CD4^+$ T cells in the absence of an efficient immune response in early infection results in large-scale virus production within the regional lymphoid tissues. As a consequence, free virus and virus-infected cells will leave the lymph node by the efferent lymphatics to infect lymph node stations further downstream and to enter the blood. This leads to generalized infection of all organs, including the central nervous system (CNS). The SIV model has shown that this initial propagation is very rapid: infection of DC in the lamina propria of the vagina and the regional lymph nodes can be detected within 2 days,

and plasma viremia was demonstrated 5 days after inoculation (Spira et al., 1996).

Acute Phase and Chronicity

Investigations in the SIV model also have shown that there is an early, dramatic effect of the virus on the immune system located in the gastrointestinal tract (Johnson and Kaur, 2005; Veazey and Lackner, 2005). The gut-associated lymphoid tissue (GALT) harbors the majority of the body's lymphocytes compared with the peripheral blood, which contains only 2% of these cells. It consists of organized lymphoid tissue (Peyer's patches and solitary lymphoid follicles) as well as large numbers of activated memory T lymphocytes diffusely distributed throughout both the intestinal lamina propria and epithelium. Due to the constant exposure to a myriad of food and microbial antigens, a major fraction of GALT $CD4^+$ T cells are activated and well differentiated with a memory phenotype. Furthermore, the gastrointestinal mucosa is in a state of constant physiological inflammation characterized by high expression levels of proinflammatory, HIV-1-stimulatory cytokines. During the first few days of infection there is a massive infection of $CCR5^+$ $CD4^+$ memory T lymphocytes by SIV, which results in the elimination of 60 to 80% of these cells within days (Veazey et al., 1998; Li et al., 2005; Mattapallil et al., 2005). As most $CCR5^+$ $CD4^+$ memory T lymphocytes of the body are located in the GALT, this wipes out 30 to 60% of the total of these cells, notably without a similar manifestation in the blood or the lymph nodes. Similar to the SIV model, studies in HIV-1-infected patients also have shown an early, rapid, profound, and persistent loss of intestinal $CCR5^+$ $CD4^+$ T cells (Brenchley et al., 2004; Mehandru et al., 2004). The early elimination of $CCR5^+$ $CD4^+$ T cells notably also includes HIV-specific $CD4^+$ T cells, which are lacking in disease progressors while being preserved in both adult and pediatric long-term nonprogressors (Rosenberg et al., 1997; Chakraborty et al., 2005). Thus, the first days and weeks of the infection may be at least as decisive for the destruction of the $CD4^+$ memory T cells, which is the hallmark of AIDS, as are the pathogenetic mechanisms during the subsequent protracted chronic stage.

Acute infection is thus the time point at which a large proportion of memory T helper cells are infected and eliminated. A small minority of surviving infected $CD4^+$ $CD45RO^+$ T lymphocytes (estimated at less than 10^6 cells) remain in, or return to, a stage of nonactivation and proviral latency (Chun et al., 1998; Schacker et al., 2000). Establishment of proviral latency in these long-lived cells is the strategy by which HIV has so far resisted all therapeutic eradication attempts (Finzi et al., 1997; Wong et al., 1997; Finzi et al., 1999).

Virus production in the lymphatics, notably also the GALT, continues during all phases of infection (Biberfeld et al., 1986; Cameron et al., 1987; Tenner-Racz et al., 1988; Embretson et al., 1993; Pantaleo et al., 1993). Monocytes and macrophages may also be an important source of infectious virus, especially after depletion of $CD4^+$ T cells in advanced disease (Orenstein et al., 1997; Igarashi et al., 2001). Virus produced in the lymphoid tissues interacts with HIV-specific antibodies, resulting in immune complex formation. These complexes then pass through the follicular DC network of the lymphatics, where they become trapped. Trapped virus remains infectious even in the presence of neutralizing antibodies and has a half-life of about 2 weeks (Heath et al., 1995; Simon and Ho, 2003).

Figure 5 summarizes the virologic and immunologic course of acute and chronic HIV infection. Hematologic dissemination from the regional lymphoid tissue draining the entry port leads to infection of all lymphoid tissues in the body, notably the GALT. Replication of HIV within the lymphatics, which harbor 98% of the total number of lymphocytes in the body, causes, in the absence of a specific immune response, a rapid increase in the production and release of viral particles and number of virus-infected cells. In the blood, this is manifested as a concomitant burst in cell-free or cell-associated infectious virus, particle-associated viral RNA, p24 antigen, and cell-associated viral RNA or DNA (Clark et al., 1991; Daar et al., 1991; Graziosi et al., 1993; Piatak et al., 1993; Koup et al., 1994). Concentrations of viral RNA in plasma may vary widely, from 10^4 to more than 10^7 copies/ml (Schacker et al., 1998). The earliest virus population observed following HIV transmission is most frequently of the R5 phenotype and genotypically very homogeneous (Zhu et al., 1993; Delwart et al., 1994; Zhu et al., 1996), even after exposure to an inoculum of mixed R5-X4 phenotype (Cornelissen et al., 1995). The predominance of R5 viruses in early HIV infection may be due to selective pressure exerted by selective transepithelial transport mechanisms like transcytosis (Meng et al., 2002) or by DC, which express CCR5 but not CXCR4. Transmission of X4 viruses, however, also has been demonstrated in about 15% of early infections (Roos et al., 1992).

Severe primary HIV infection is characterized by an initial lymphopenia followed by $CD8^+$ T lymphocytosis and inversion of the CD4/CD8 ratio. Subsequently, the $CD8^+$-T-cell count gradually returns to normal, whereas the CD4/CD8 ratio remains inverted because of a relatively small number of $CD4^+$ lymphocytes. Primary infection is followed by a prolonged and severe cellular hyporesponsiveness to both mitogens and antigen (Pedersen et al., 1990; Sinicco et al., 1990).

Virus levels decrease with the onset of the antiviral immune response, namely, the production of HIV-specific cytotoxic T lymphocytes (CTLs) (Koup et al., 1994; Pantaleo et al., 1994; Connick et al., 1996). During this initial cellular response, up to 6% of the $CD8^+$ T cells may represent HIV-specific CTLs (Roos et al., 1992; Yang et al., 1996; Borrow et al., 1997). Studies of SIV-infected macaques in which CD8 T cells were temporarily ablated by infusion of a CD8-specific monoclonal antibody have also demonstrated the importance of these cells in lowering the viral load in both primary and chronic infection (Jin et al., 1999; Schmitz et al., 1999). Moreover, after seroconversion, antivirus antibodies that bind to virus particles and to which complement is fixed may increase virus retention on follicular DC that carry complement receptors at high density and thus can retain large quantities of complexed infectious virions (Embretson et al., 1993; Pantaleo et al., 1993; Heath et al., 1995). In agreement with this, the viral RNA load in early chronic HIV infection is high in lymphoid tissues but low in plasma (Pantaleo et al., 1998).

After the initial peak, the virion concentrations in blood are, at least in some patients, stabilized on individually different levels. This so-called "set point" or "inflection point" is strongly associated with disease outcome (Jurriaans et al., 1994; Henrard et al., 1995; Mellors et al., 1996; Schacker et al., 1998). The set point is the equilibrium that results from the interplay of viral, host cell, and immunological factors and is usually reached within a few months to 1 year of infection (Kaufmann et al., 1998; Schacker et al., 1998). Viral titers in plasma subsequently increase only slowly for a long time, corresponding to clinical latency. During this time, the $CD4^+$-T-cell count decreases continuously at an individually different but constant rate. In the lymphatics,

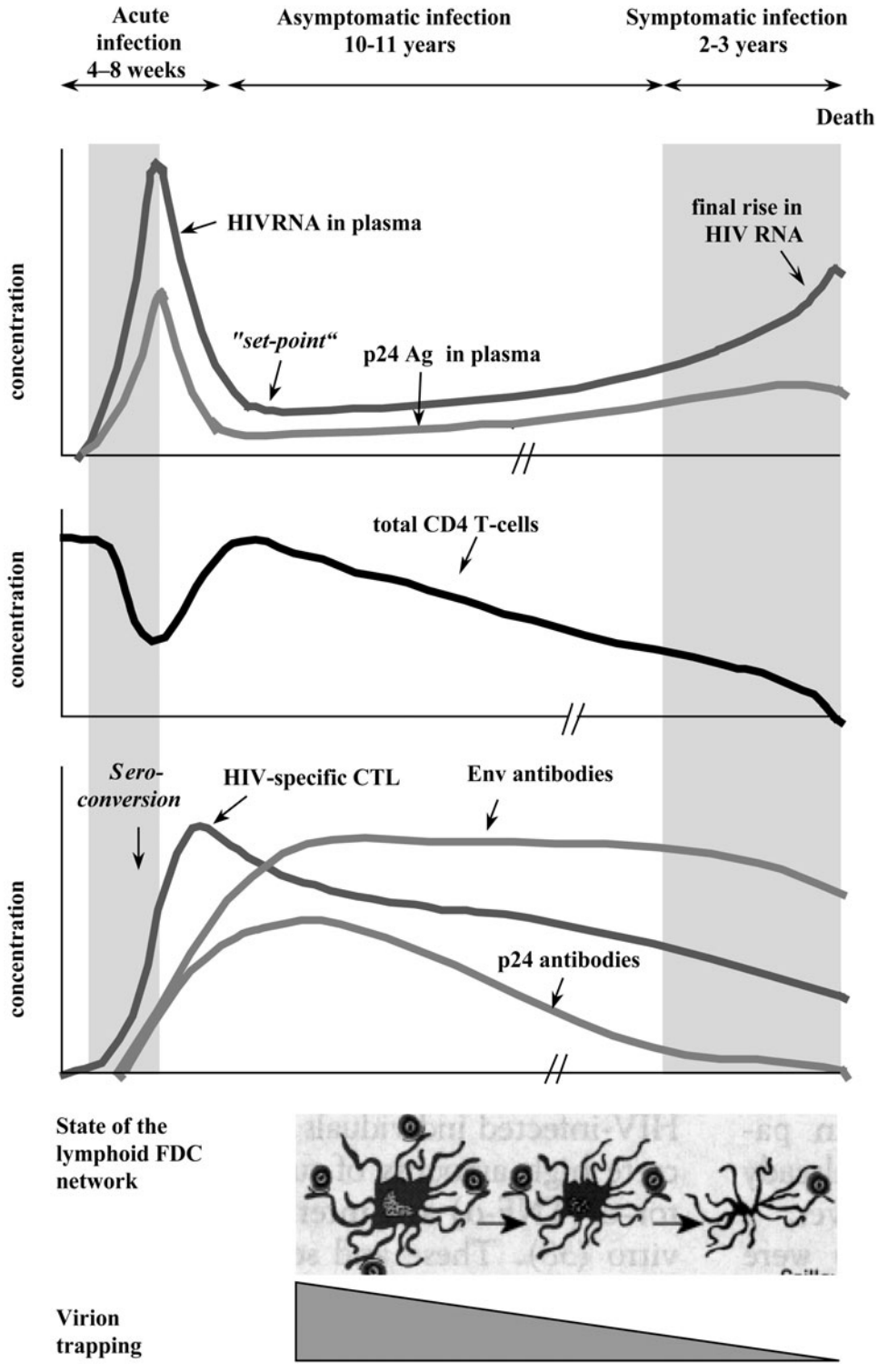


FIGURE 5 Virologic and immunologic parameters in the typical course of HIV infection.

there is a continuous, progressive destruction of the follicular DC (FDC) network, leading to the complete loss of the regular lymph node architecture (Fig. 5, bottom). A marked increase in the level of viral RNA in plasma is seen in advanced immunodeficiency, when the $CD4^+$-T-cell count has dropped to below 200/μl. This has been interpreted as a final complete breakdown of the mechanisms that previously maintained a certain control over virus replication. The destruction of the follicular DC network may also contribute, as it leads to a decreased retention of virions; hence,

more virus will reach the peripheral blood (Fauci, 1993). Frequently, the final increase is also preceded by an emergence of X4 viruses (Schellekens et al., 1992; Koot et al., 1993).

Dynamics of HIV Replication In Vivo

The availability of antiretroviral drugs which interrupt virus replication and experiments involving plasmapheresis have permitted determination of the dynamics of virus replication (reviewed in Simon and Ho, 2003). The half-life of virus in plasma is 56 min on average. To keep the virus concentration in an equilibrium, at least 10^{10} virus particles must be produced per day. About 93 to 99% of the virus in the blood plasma of untreated patients originates from activated $CD4^+$ T lymphocytes that get infected, produce virus, and die with a half-life of only 0.7 ± 0.2 days (so-called productively infected $CD4^+$ T lymphocytes). An additional 1 to 7% of the virus in plasma originates from longer-lived cells (replication in monocytes or macrophages, release of surface-bound virus from DC) that have a half-life of 14 ± 7.5 days. Less than 1% of the virus in plasma is produced by latently infected $CD4^+$ T cells, which become activated and then start producing virus. This last compartment has a very slow decay rate. Its half-life is estimated at 6 to 44 months (Finzi et al., 1997; Wong et al., 1997; Finzi et al., 1999), or it may not even decay at all (Siliciano et al., 2003). Eradication of this compartment will not be possible without measures that activate the virus from its state of latency.

HIV-Associated Diseases

Acute Retroviral Syndrome

The first clinical manifestations may appear a few days to a few weeks after infection with a transient condition frequently called acute retroviral syndrome. It is found in 50 to 70% of infected patients and is characterized by clinical signs of immune activation and multisystem dysfunction. Patients frequently seek medical attention due to a flu-like or infectious mononucleosis-like disease with fever, generalized lymphadenopathy, sore throat, arthralgia, myalgia, fatigue, rash, and/or weight loss. The rash consists of a maculopapular exanthem, especially of the trunk, with occasional transition into a papulovesicular appearance. Lesions of the oral mucosa, often aphthous, may also occur. Occasionally, diarrhea, pancreatitis, mild disturbance of hepatic functions, bacterial sepsis, thrombocytopenia, epiglottitis, lymphocytic alveolitis, or self-limiting neurological disorders like meningitis, encephalitis, polyneuropathy, or myelopathy are found. Opportunistic infections normally seen only in advanced immunodeficiency, such as esophageal candidiasis, may be present. These symptoms typically resolve within 5 to 30 days.

Clinical Latency

Acute infection is followed by a long stage of disease-free clinical latency. The median time to AIDS in untreated adult patients is estimated at 10 to 11 years. The incubation time varies considerably. In the 5 to 10% of patients who are rapid progressors, AIDS develops within 2 to 3 years after infection. At the other end of the spectrum, 5 to 10% of patients are nonprogressors and are free of symptoms after 7 to 10 years with stable, although lower than normal, CD4 T-cell counts. In maternally transmitted pediatric infection, disease progression follows a bimodal distribution, with a subgroup of children progressing rapidly to AIDS at a median age of approximately 5 months and 20% of infected children developing AIDS within 12 months (Downs et al., 1995). The mean time from birth to the stage of severe symptoms was estimated at 6.3 to 6.6 years, and the time to death was estimated at 6.3 to 9.4 years (Barnhart et al., 1996; Pliner et al., 1996). Although children thus usually develop moderate symptoms by the second year of life, they do not progress further for a long time (Barnhart et al., 1996).

TABLE 2 CDC 93 classification for HIV infections

$CD4^+$ cell category	Clinical category		
	A[a]	B[b]	C[c]
1 (≥500/μl; ≥29% of lymphocyte count)	A1	B1	C1
2 (200–499/μl; 14–28% of lymphocyte count)	A2	B2	C2
3 (<200/μl; <14% of lymphocyte count)	A3	B3	C3

[a]Asymptomatic, acute (primary) HIV or persistent generalized lymphadenopathy.
[b]Symptomatic, not category A or C conditions (see Appendix 1).
[c]AIDS-indicator conditions (see Appendix 2).

AIDS

The relentless production of HIV proteins, maintained by continuous viral replication in productively infected cells, and the ensuing elimination of host cells over many years finally lead to the destruction of the immune system, which is clinically manifested by opportunistic infections and tumors (Appendices 1 and 2). In addition, infection of the CNS may lead to distinct HIV-associated diseases, including the HIV-associated dementia complex, vacuolar myelopathy, and sensory neuropathy (Petito et al., 1999; Price, 1996).

Several disease classifications have been introduced since the first description of AIDS in 1981 (Gottlieb et al., 1981). The currently used Centers for Disease Control and Prevention (CDC) 1993 classification (Table 2 and Appendices 1 and 2) is based on a combination of clinical and CD4 T-cell count categories that defines nine mutually exclusive stages (Centers for Disease Control, 1992).

Diagnosis of HIV Infection

The two principal questions in HIV diagnostics are whether a person is infected and, if infected, how actively the virus is replicating. The susceptibility of a patient's virus to antiretroviral drugs has emerged as another question of eminent practical importance.

HIV infection can be detected by a variety of tests. Assayed virus components include proteins, especially p24, which can be measured by immunological tests; RT, whose enzymatic activity can be detected by functional tests; and viral DNA or RNA, which can be identified by molecular tests. Most frequently, however, HIV infection is diagnosed by tests that assess whether an individual's immune system has produced an HIV-specific immune response. Since retroviruses are known to establish infections that persist for life, demonstration of an HIV-specific immune response, if it is consistent and directed against various viral antigens, can be trusted to reflect ongoing infection. Thus, testing for HIV-specific antibodies is still the mainstay of HIV diagnostics, at least in adults. In infants, only testing for virus components allows early diagnosis or exclusion of infection.

The diagnosis of HIV infection relies on commercially available test kits. Competition among manufacturers and strict evaluation and control by regulatory authorities have led to a large number of excellent well-standardized commercial

diagnostic products of high sensitivity and specificity which provide a continuously high standard of quality. They are usually better and yield more consistent results than research procedures developed in diagnostic laboratories. Good commercial tests are therefore strongly recommended. Using unregistered tests for screening or for certain types of supplemental testing is unlawful in many countries. In the United States, refer to http://www.fda.gov/cber/products/testkits.htm for the actual list of U.S. Food and Drug Administration (FDA)-approved commercial diagnostic tests. Commercial tests for diagnostic use in Europe need to be Communauté Européenne (CE) marked.

Only very general descriptions of procedures are given in the following sections, since commercial test kits all contain detailed step-by-step instructions. For procedures that are not commercially available, the reader is directed to the referenced literature. The intent is to guide the reader through the multitude of available procedures and to discuss their strengths and weaknesses.

Screening for HIV Infection, Early Infection Window Periods

HIV-specific antibodies are produced within a few weeks after infection. The time to positivity in screening tests (i.e., to seroconversion) may be influenced by the phenotype of the infecting virus, the infectious dose, the transmission mode, and the sensitivity of the assay.

In a study based on the first generation of HIV antibody screening assays, developed more than 2 decades ago against subtype B, seroconversion was estimated to occur on average 45 days after infection; with 95% certainty, the window period for 90% of individuals was less than 20 weeks (Petersen et al., 1994). The usefulness of more recently developed tests in reducing the average window period has since been estimated as follows: third-generation anti-HIV-1 and -2 enzyme immunoassays based on detection of antibodies, –20.3 days (95% confidence interval [CI], 8.0 to 32.5); use of p24 antigen or PCR for proviral DNA , –26.4 days (CI, 12.6 to 38.7); and PCR for viral RNA in plasma, –31.0 days (CI, 16.7 to 45.3) (Busch et al., 1995). With modern third-generation antibody-screening assays, half of the infected individuals should become antibody positive within 3 weeks after infection. Most of the other half should become positive within 2 months, but 5% still seroconvert more than 6 months after infection. It is important to realize that the use of tests for viral RNA, DNA, or p24 in such patients reduces the long diagnostic window periods only insignificantly by 1 to 2 weeks (Busch et al., 1997).

Compared to the 3-week median window of third-generation antibody assays, p24 antigen testing or the use of fourth-generation combination assays that detect both HIV antibodies and p24 antigen reduces the window by a further 5 days (i.e., to 16 days). Finally, the most sensitive test currently available, a test for HIV-1 RNA with a detection limit of 50 copies/ml, reduces the median window length by a further 7 days (i.e., to 9 days). The viral load at which p24 antigen would be detected was estimated by regression analysis at 10,000 copies/ml (CI, 2,000 to 93,000) and the HIV replication rate at 0.35 log copies/ml/day, corresponding to a virion doubling time in the preseroconversion phase of 20.5 h (Fiebig et al., 2003). Note again that the 9-day window period for HIV-1 RNA tests is a median and that, as mentioned above, the most sensitive tests for HIV-1 RNA do not significantly shorten the window period of patients with late seroconversion. Late seroconversion cannot be excluded by a negative HIV-1 RNA test.

Formats of Screening Tests

There are numerous commercial HIV tests for screening, and it may be difficult to recognize the advantages and disadvantages of a particular test based on the information given by the manufacturer and without systematic comparison (Courouce et al., 1999). An overview of different test formats and their properties is given in Fig. 6.

The most important kit formats used for HIV antibody screening are the indirect binding assay, the antibody capture assay, and the double-antigen sandwich (DAGS) assay. Indirect binding assays comprise the so-called first-generation enzyme-linked immunosorbent assays (ELISA), which are based on purified viral lysate, and so-called second-generation tests which utilize recombinant antigen or synthetic peptides usually representing Gag and transmembrane (TM) protein. The first-generation indirect binding assay format also applies to immunofluorescence tests and Western blot (WB). Line immunoassays (LIA), which use recombinant proteins and synthetic peptides, may be considered second-generation tests. Antibody capture assays usually employ recombinant proteins; their principle is that of an indirect binding assay reversed. DAGS assays, frequently also called third-generation assays, usually employ recombinant antigen. Particle agglutination assays may be considered a variant of DAGS assays because, to generate a positive signal, an antibody molecule must react with at least two antigen molecules, each located on a separate gel particle.

Although all of these tests detect antibodies, they vary in their precise diagnostic questions and answers. Indirect binding assays and antibody capture assays verify, by binding the sample's HIV-specific antibodies to an immunoglobulin (Ig)-specific reagent, that the component that causes reactivity in such a test is indeed an Ig. In contrast, the identity of a component causing reactivity in a DAGS assay remains uncharacterized; the only information provided is that it is capable of linking solid-phase HIV antigen with liquid-phase tracer antigen.

The different kit formats are affected in different ways by diagnostic challenge situations. One such challenge is antigenic variation. The virus with which a patient is infected may exhibit antigens which differ considerably from the antigens used in the test. Consequently, the patient's antibodies may not bind well to the test kit's antigens, and if the antibody titer is low, a false-negative result may be generated. This type of problem was recognized when antibodies induced by HIV-2 infection were not well recognized by screening kits based on HIV-1 antigens alone. This led to the inclusion of HIV-2 components, usually of the TM protein, in the kits. A similar problem was recognized when group O viruses were discovered, leading to inclusion of group O antigens into all CE marked test kits in Europe (De Leys et al., 1990; Gurtler et al., 1994). DAGS assays are the assays most affected by antigenic variation, because an antibody molecule must bind at least two antigen molecules to generate a signal. Such double binding is unlikely if the kit's antigens and the patient's antibodies do not fit. Moreover, endogenous soluble viral antigen present in the serum sample may compete with the test's antigens for free binding sites on HIV-specific antibodies. IgG, which contains only two antigen binding sites per molecule, is most strongly affected, since a single endogenous antigenic molecule suffices to abolish detection of an IgG molecule in a DAGS assay. In contrast, in indirect binding and antibody capture assays, antibodies need bind only a single antigen molecule to generate a signal.

Another diagnostic challenge is early seroconversion. Antibodies in this phase are restricted to a few viral antigens

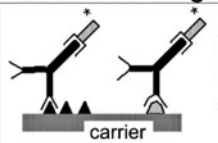
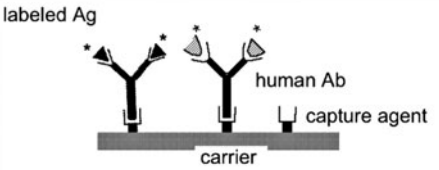
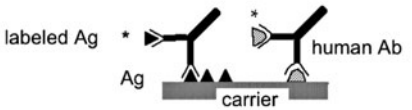
	Indirect Binding Assay	Antibody Capture Assay	Double Antigen Sandwich Assay
TEST PRINCIPLE	 Ag bound to carrier serves as the target for the patient's Ab. Bound Ab is detected by 2nd Ab. Early selection for Ag-specific Ab. ***"1st generation test"*** EIA, IF, WB Ag from viral lysate, contains in addition to the full range of viral Ag (▲) many cellular Ag (△). ***"2nd generation test"*** EIA, LIA Restricted number of recombinant or peptide viral Ag permits increased concentrations of selected viral Ag on plate; less nonspecific Ag (expression system) or none (synthetic peptides).	 "Indirect Binding Assay reversed" Capture agent (anti-hu Ig, protein A, G) specific for human Ig (frequently used for IgM, IgA detection) immobilized on carrier. Human Ig of all specificities bound. Labelled Ag binds to Ab of corresponding specificity. Selection for virus-specific Ab in last step. Labeled viral Ag may be lysate-derived, recombinant, or a synthetic peptide.	 ***"3rd generation test"*** Ag bound to carrier acts as target for patient's corresponding Ab. Bound Ab is detected with same, labelled Ag added in solution. Early and late selection for specific Ab leads to increased Ag specificity, but this still depends on purity of Ag used (recombinant and/or synthetic peptide). Particle agglutination follows similar principle!
MESSAGE OF A POSITIVE RESULT	There is a reactive ***antibody.*** Ag to which Ab binds **may** be viral (or a cellular/bacterial contaminant).	There is a reactive ***antibody*** (of a certain Ig isotype) (otherwise same as indirect binding assay).	There is ***something*** that reacts with the Ag. (Whether this is an Ab remains open — these tests are not immunological in the strict sense!)
Challenge # 1: Ag Variation (e.g. group O): a different, though structurally related immunogen (▲) induces Ab with low avidity to Ag used in test (▲).	Bound Ab molecules still well detected by 2nd Ab. First generation less affected: at least one of the several viral Ag probably recognized by patient's Ab. 2nd generation: sensitivity possibly impaired, due to more restricted specificity of viral Ag presented on the plate.	Binding of Ab to capture agent unaffected. Each bound Ag-specific Ab has double chance that an Ag molecule will bind. Soluble Ag can be added at higher concentration than on carrier. Additional advantage with use of viral lysate. Probably the test format affected least.	Each Ab molecule must make and maintain contact with at least 2 Ag molecules. Low affinity effect is thus magnified. If antigenemia is present, no free binding site may be available for the labeled Ag. Altogether probably the format affected most. Remedies: add variant Ag to both solid and liquid phase; add labelled 2nd Ab to human Ig.
Challenge # 2: Seroconversion: low Ab concentration against a restricted number of Ag; most Ag-specific Ab of IgM isotype; viremia, Ag-emia.	1st generation: Envelope Ag relevant in early seroconversion not present at sufficiently high concentration in viral lysate on plate. 1st/2nd generation: bound IgM may be detected suboptimally by 2nd Ab (if it does not recognize μ-chains). Due to lower specificity than achieved with double Ag sandwich format, signal amplification can not be as efficient; thus, limit of detection is increased/ analytical sensitivity lowered.	No early selection for Ag-specific Ab and limited Ig capture capacity lead to inefficient binding of rare Ag-specific Ab, particularly if they are of IgM or IgA isotype. Probably the test format most affected.	Very efficient linking of the test's solid phase and liquid phase Ag by IgM, even if some of IgM's Ag-binding sites may already be occupied by the patient's own Ag. Currently best-suited format for antibody detection in seroconversion. Are for screening still inferior to tests which simultaneously detect Ag (***"fourth generation tests"***).

FIGURE 6 Kit design and test performance of HIV screening tests. Synopsis of the most frequently used test formats, their principles, the meaning of positive results, and performance in two typical problem situations. Ag, antigen; Ab, antibody; hu, human.

(usually envelope and p24) and are of low titer and low affinity, and the dominating isotypes are IgM and possibly IgA. In addition, these antibodies may be partially complexed with HIV antigen, which is usually present at high concentration in primary HIV infection (Fig. 5). In this situation, it is important that the test provides a high concentration of that antigen that is best recognized. This goal is more easily achieved with recombinant proteins than viral lysate. Furthermore, the test must select for the few HIV-specific antibodies present in the bulk immunoglobulin; this is impossible with antibody capture assays that bind Ig of all antigenic specificities. In addition, the test should detect IgM because, in the presence of antigenemia, its pentameric structure with a total of 10 antigen binding sites is most likely to have several sites remaining accessible. The best assay in this situation is the DAGS assay: it initially selects for HIV-specific antibodies (binding to solid phase) and does not discriminate against non-IgG isotypes. The first test based on this principle was the particle agglutination assay, which was introduced in the mid-1980s, i.e., long before third-generation ELISA were developed. This test performs remarkably well in seroconversion panels, and due to the broad spectrum of antigens present in the viral lysate, it also has a broad detection range for antigenic variation (Constantine et al., 1994; Vercauteren et al., 1995; Poljak et al., 1997; Lien et al., 2000).

The practical relevance of these considerations is shown when the performances of different kits with seroconversion panels are compared. Among 23 different commercial kits whose performance on at least 15 different commercially available seroconversion panels was compared by the Swiss Federal Office of Public Health, the 17 DAGS assays were the most sensitive and occupied ranks 1 to 15, 17, and 18. The four indirect binding (second-generation) assays occupied ranks 16, 19, 21, and 22, and the two antibody capture assays ranked 20th and 23rd (J. Schüpbach, unpublished data). Seroconversion panel comparisons also demonstrated the inferior sensitivity of immunofluorescence tests and WB, which ranked at the end together with other first-generation indirect antibody binding assays (see also Busch and Satten, 1997; Thorstensson et al., 1998). Assessment of the performance in seroconversion panels followed by revocation of approval for the 10 to 20% least sensitive kits, is one of the most powerful—though obviously underused—instruments by which regulatory agencies could guarantee a continuous further technical improvement of diagnostic tests (Schupbach, 1996).

Fourth-Generation Screening Tests

Several companies now offer kits that detect both antibodies and antigen (fourth-generation tests), and in many European countries, the use of these products for HIV screening performed in diagnostic laboratories has become mandatory. In seroconversion panel analysis, these kits now rank first among all screening tests even if their detection of antibodies is based on the insensitive antibody capture format. The average gain in time to detection compared with third-generation kits is 3 to 5 days (Gurtler et al., 1998; Weber et al., 1998; Laperche et al., 2000; Ly et al., 2001). The use of such tests for screening is strongly recommended because individuals in the antigen-positive stage of preseroconversion have a high viral load and are particularly infectious (Fig. 5).

Rapid Tests and Use of Alternative Specimens

Rapid tests can be performed with minimal or no laboratory equipment; they yield results within 30 min. Such tests may be useful in certain situations, e.g., in assessing the risk of HIV transmission in needlestick injuries and similar exposures to possibly HIV-contaminated materials, organ donations, or whenever a laboratory test result may not be available quickly. Rapid tests may be of different formats, including DAGS, indirect binding, Ig capture, agglutination, or chromatographic assay. The diagnostic sensitivity of some of these tests seems somewhat inferior to third-generation ELISA-based antibody tests, especially in seroconversion panels (Kuun et al., 1997; Vallari et al., 1998; Giles et al., 1999). Others, however, exhibit a comparable diagnostic sensitivity and specificity, even during seroconversion, and can therefore be recommended for certain diagnostic settings (Giles et al., 1999; Kelen et al., 1999; Palmer et al., 1999; Zaw et al., 1999; Phillips et al., 2000; Ketema et al., 2001).

Many persons infected with HIV are not tested until they develop symptoms of AIDS. Up to one-third of patients receive their HIV diagnosis within 2 months of progression to AIDS. The hope that such individuals could be motivated to be tested earlier has led to new testing strategies, particularly in the United States. These now recommend routine, "opt-out" testing in all health-care settings (Branson et al., 2006). The shift in testing strategy also has led to the use of new test systems believed to be more attractive to the client. They include home collection test systems, in which sample collection devices are ordered by phone and delivered by express courier. Blood is collected by finger pricking onto filter paper and sent to a designated laboratory for screening. Such testing systems have good sensitivity and specificity; collecting a sufficiently large specimen may be the biggest problem, affecting 7 to 10% of the users. As an alternative, testing systems for other specimens, such as oral fluids or urine, also received FDA approval (reviewed in Mylonakis et al., 2000). Excellent sensitivity and specificity were reported in studies involving oral fluids collected from postseroconversion individuals (Saville et al., 1997; Wisnom et al., 1997; Granade et al., 1998; Martinez et al., 1999). This also applies to FDA-approved test systems for urine samples (Urnovitz et al., 1997). Very recently, the FDA has approved a rapid test system for oral fluids, whole blood, or serum which is so easy to perform that testing at the point of care with a return of the result within 20 to 40 min has become possible. Extended studies of this device have reported a sensitivity and specificity comparable to that of other enzyme immunoassays (EIAs) (Delaney et al., 2006; Wesolowski et al., 2006). The sensitivity of test systems utilizing specimens other than blood in early seroconversion remains untested, as standardized materials comparable to seroconversion panels are not available. The use of such alternative tests in recent exposure settings should therefore be avoided. True home tests, which would be sold to the public, have not been approved by the FDA or the health authorities of other countries, and their safety cannot be guaranteed.

Supplemental Testing

Antibody Tests—WB and LIA

WB was introduced into HIV testing by the author in 1984 (Sarngadharan et al., 1984; Schupbach et al., 1984), was proposed for systematic confirmation of reactive screening results in 1985 (Schupbach et al., 1985), and has remained a principal confirmatory tool worldwide (Mylonakis et al., 2000). Over the years, however, it has also become clear that, in contrast to the continuously improved screening tests, WB has remained a first-generation test with certain well-known flaws: the sensitivity in seroconversion panels

is clearly inferior to that of third- and fourth-generation screening tests. WB is also prone to detect cross-reactive antibodies, which results in a high rate of indeterminate results.

A single improvement, the use of recombinant proteins and synthetic peptides for the production of the strips, has been realized by some manufacturers. When recombinant proteins and peptides are used entirely instead of viral lysate, strips can be produced as LIA in which selected antigens are applied as distinct lines and at defined, optimal concentrations. In format, such assays are comparable to second-generation screening EIAs and may thus be considered "second-generation WB." One such assay, the Inno-Lia HIV I/II Score, is increasingly used in countries outside the United States. It contains 7 HIV antigen bands (sgp120 [including group O peptides], gp41, p31, p24, and p17 of HIV-1; sgp105 and gp36 of HIV-2), which are coated as discrete lines on a nylon strip with plastic backing. As each test strip also contains 3 quantitative internal standards, a semiquantitative ranking of the different antibody reactions into 6 intensity scores is possible. This enables a standardized interpretation of test reactions which, unlike for WB, is not only based on the presence of reactions (yes/no), but also their intensity. This LIA provides excellent confirmation of HIV infection and is superior to WB for differentiating between HIV-1 and HIV-2 infection (Pollet et al., 1991; Walther et al., 1995).

Early identification of HIV-2 infection is important with regard to both virus load quantification and the choice of effective antiretroviral treatment (ART). None of the virus load assays approved for patient monitoring can reliably quantitate HIV-2, and HIV-2 virus loads in symptomatic patients may be severely underestimated. Furthermore, HIV-2 is naturally resistant to nonnucleoside RT inhibitors (NNRTI) and some other antiretroviral drugs effective against HIV-1. HIV-2 infection must therefore be diagnosed early to prevent suboptimal disease monitoring and start of ineffective treatment regimens leading to resistance.

WB and LIA are more prone to problems with carryover contamination than are most screening assays. Use of the convenient multichannel troughs for incubation of the strips presents a certain risk. Contamination with minute volumes of a strongly positive serum may lead to faint Env bands, even if the dilution is up to 10^6-fold. While intra-assay contamination can be ruled out by repeating the assay in an isolated test chamber, repeat testing will not identify contamination within the specimen tube. Touching the wet inner side of a specimen tube lid with the gloved fingers may carry enough material to the lid of a subsequently opened tube to result in faint WB reactivity to Env antigens. The probability of such events depends on the proportion of strongly positive sera among the specimens tested by a laboratory and on how many times a specimen tube is opened. To minimize this risk, handling and testing of samples from known HIV-positive patients together with diagnostic samples should be avoided, and gloves that have become contaminated with specimen must be changed immediately. It should also be recognized that samples with initial borderline results carry an increased risk of contamination, since these tubes are opened repeatedly for supplemental testing. This results in a higher cumulative risk of contamination. Alarm bells should ring when a sample with borderline or low positive results in screening is faintly reactive in WB. It may be an early seroconversion sample, but it may also be the result of contamination. The contamination problem is a strong reason why WB interpretation should follow the most stringent and not the most sensitive guidelines.

WB, and to a lesser degree, LIA still have a relatively high rate of indeterminate results (Pollet et al., 1991). This shows that indeterminate WB reactions are usually caused by antibodies that cross-react with viral rather than with cellular proteins. Indeterminate WB results have been described for patients with autoimmune disorders, particularly systemic lupus erythematosus; after infections with certain viruses, including herpes simplex virus type 1 or cytomegalovirus; or after vaccination against influenza or rabies virus (Guan, 2007). For the latter, epitopes related to HIV have been implicated. Such information is, however, of little practical value, and the origin of indeterminate WB reactions usually remains obscure. In spite of all these flaws, a WB or LIA with a "full-house" pattern of reactive antibodies probably remains the most convincing laboratory evidence for an HIV infection. When reactive bands are few and their intensities are low, interpretation is hazardous, and a diagnosis must not be based on WB or LIA alone.

WB Interpretation Guidelines

In an attempt to render WB more sensitive, the Association of State and Territorial Public Health Laboratory Directors and the CDC (ASTPHLD-CDC) issued interpretation recommendations which request antibody reaction to any two of three antigen bands, including gp120/160 (considered to be one antigen), gp41, and p24 (Centers for Disease Control, 1989). Since most of the gp160 and gp120 bands on WB are not due to the Env precursor or the surface (SU) protein but instead represent tetramers or trimers of gp41 (Pinter et al., 1989), reaction with gp120-gp160 and gp41 bands may be based on reaction with a single protein, TM, and is thus inherently unsafe. The same is true for the very similar recommendations by the Consortium for Retrovirus Serology Standardization, the only difference being that p24 may be replaced by p31 (*pol*). Similarly, the World Health Organization recommendation specifies any two of gp160, gp120, and gp41 (Anonymous, 1990). This means, practically, that TM-reactive antibodies must be present at a concentration sufficient for detection of not only the strongest but also the second-strongest TM band. The strongest TM band is usually the largest antigen, i.e., gp160, which migrates least far in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis-based separation of proteins and thus is the sharpest and best-detected band. Depending on the manufacturer, either gp120 or gp41 may be the second strongest band. Due to varying degrees of glycosylation, gp41 migrates in sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a very diffuse band. Reactive antibodies thus generate a signal that is much less easily recognized than if the same antibodies bind to the sharp gp160 band.

Due to the propensity of WB to detect cross-reactive antibodies, a combination of Env and p24 bands is not sufficiently stringent for confirmation. WB analysis of 100 screening-negative but otherwise unselected Swiss blood donors showed an isolated reaction with p24 in 9% and with gp160 or both gp160 and gp120 in 3%. The likelihood of a chance combination leading to "confirmed positivity" in a healthy donor would thus be 0.09×0.03, or 0.0027, i.e., 1 in 370 (Schupbach et al., 1990). We and others (Healey and Bolton, 1993) have observed several cases that satisfied the ASTPHLD-CDC criteria for WB positivity and kept this pattern essentially unchanged over the years but were negative in long-term follow-up in all direct tests for HIV components, including virus culture, regular PCR for HIV DNA and RNA, and an ultrasensitive sequence capture-PCR test "Mega-PCR" enabling detection of a few provirus copies in as much as 100 to 500 μg of DNA (Boni et al., 2004b). Representative WB results of such a case are shown in Fig. 7.

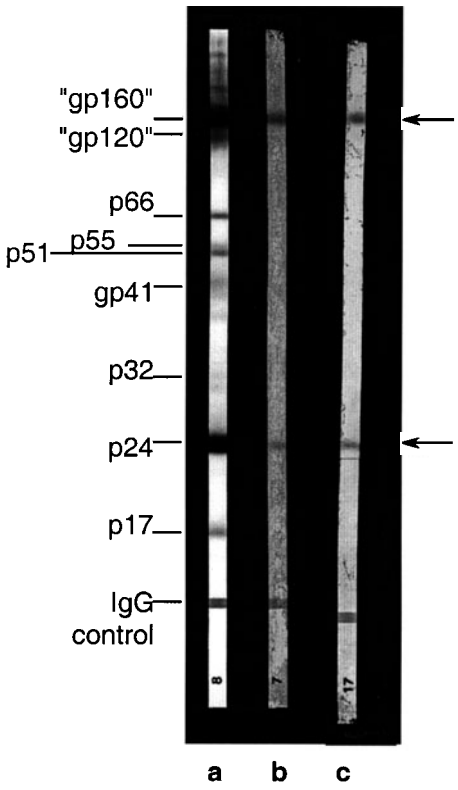


FIGURE 7 Example of a false-positive HIV-1 WB interpretation according to ASTPHLD-CDC or Consortium for Retrovirus Serology Standardization guidelines. Lanes: a, weakly positive control; b, sample from a healthy individual exhibiting weak reaction with gp160, gp120 (very weak), and p24 (this sample was taken 3.5 months after an initial sample with the same pattern [data not shown]); c, sample from the same individual taken 1 month after the initial sample. p24 antigen with signal amplification-boosted ELISA was negative in all three plasma samples. PCR for viral DNA was negative in PBMC from the samples in lanes b and c, and RNA was negative in the sample in lane b. Culture with PBMC depleted of CD8 T cells from the sample in lane b was negative for p24 antigen and RT by the PERT assay; this test was also negative with the samples in lanes b and c.

More-stringent criteria have been issued by the American Red Cross (ARC) and the FDA. ARC specifies at least one band each from Env, Gag, and Pol. The most stringent but least sensitive recommendation is that of the FDA, which specifies reaction with p24, p31, and Env.

In my opinion, only the most stringent interpretation guidelines should be applied if a diagnosis of HIV infection is established using WB as the only supplemental test. If some true cases of HIV infection are WB indeterminate by FDA guidelines, this is of no concern as long as their WB pattern is suggestive of HIV infection. This is always the case when the ASTPHLD-CDC interpretation would render these patterns positive. In almost all such cases, a safe diagnosis can be established based on supplemental tests for virus components (p24 antigen or nucleic acids). One also has to take into account that the ASTPHLD-CDC criteria were established based on a single commercial product. Meanwhile, other kits are available. Guidelines established for one particular kit and with one particular sample cohort cannot be applied to other kits or populations from other geographical regions without careful reexamination of their validity.

Another attempt to render WB more sensitive in early infection is its use in the detection of IgM. Unfortunately, such testing lacks specificity. Gag-reactive antibodies of IgM, IgA, and IgG isotype are frequently detected by WB in sera of infants born to HIV-negative mothers. Therefore, many of these reactions appear to be due to common agents unrelated to HIV (Schupbach et al., 1994).

Virus Component Tests—p24 Antigen and Nucleic Acid Tests (NAT)

In many cases, supplemental testing requires the use of tests for virus components. This applies to patients with primary HIV infection as well as those with indeterminate results in antibody tests. Diagnosis of pediatric HIV infection is also best established with virus component tests. Virus components that can be assayed include the p24 antigen, viral DNA or RNA, and RT activity. In addition, the capability of the virus to replicate can be assessed by virus isolation in cell culture. This requires a virus component test, usually an antigen test or immunofluorescence for cell-associated HIV antigen.

Commercially available tests for virus components include p24 antigen assays. CE-marked p24 antigen assays are available from various manufacturers in Europe, while in the United States, only one test is FDA approved. A CE-marked kit is available for PCR detection of HIV-1 DNA but lacks approval in the United States. NAT for HIV-1 RNA that are both CE-marked and FDA-approved are available from several manufacturers and currently include PCR for reverse-transcribed RNA (RT-PCR), nucleic acid sequence-based amplification (NASBA), which is also known as transcription-mediated amplification (TMA), and a signal-amplification procedure which involves branched DNA (bDNA) probes.

Tests for HIV p24 Antigen

Antigen tests for p24 are easy to perform and diagnostically valuable in early infection, when antigen is usually present at high titers and while HIV-specific antibodies may still be undetectable. Although NAT are increasingly used instead of p24 antigen, this test still has a place in supplemental testing because it can, in contrast to NAT, also be performed on serum, thus enabling diagnosis of primary infection in the first specimen received for HIV testing, usually serum. A highly improved version of a p24 antigen test, involving a signal amplification-boosted p24 EIA of heat-denatured plasma, also has proven to be a sensitive, specific, simple, and inexpensive solution for diagnosing pediatric HIV-1 infection in resource-poor settings, especially in subtype C epidemics (Schupbach, 2003a; Fiscus et al., 2006; Patton et al., 2006).

Tests for Viral RNA or DNA

Three fundamentally different techniques for the sensitive detection or quantification of HIV RNA or DNA are available.

PCR. In PCR, double-stranded DNA is denatured, a pair of HIV-specific oligonucleotide primers is annealed to the separated viral DNA strands, and these primers are extended by a heat-resistant DNA-dependent DNA polymerase (*Taq* polymerase). This procedure is continued for 30 to 40 cycles, each of which comprises a high-temperature denaturation, a

low-temperature primer annealing, and an intermediate-temperature primer extension (DNA synthesis) (Kwok et al., 1987). If the starting material for PCR is RNA, a cDNA must first be generated by reverse transcription. This cDNA can then be amplified by the regular procedure (Byrne et al., 1988). A further development of PCR, TaqMan real-time PCR, employs the AmpliTaq Gold DNA polymerase for PCR amplification. This enzyme also has a 5′ exonuclease activity, enabling it to cleave away a synthetic oligonucleotide called a TaqMan probe annealed to a specific sequence of the template between the forward and reverse amplification primers. The probe, which contains a fluorophor at the 5′ end and a quencher at the 3′ end, sits in the path of the enzyme as it proceeds to copy DNA or cDNA. When the enzyme reaches the annealed probe, it cleaves the probe into its nucleotides, thus releasing the fluorophor from the inhibitory effect of the quencher. Thus, the growing copy number of amplified DNA is accompanied by a likewise increasing intensity of fluorescence, which is measured continuously, thus permitting real-time observation of the amplification process. Important advantages of real-time PCR include the broad range of concentration in which the target DNA is measured precisely and the protection against carryover contamination, as both amplification and product analysis take place in a closed system.

RT-PCR test systems for both manual sample preparation and or fully automated operation are available from both Roche (Roche Molecular Diagnostics, Pleasanton, CA) and Abbott (Abbott Laboratories, Abbott Park, IL). The assays manufactured by Roche (Amplicor HIV-1 Monitor v. 1.5, Cobas AmpliPrep/Cobas TaqMan HIV-1) amplify a sequence in *gag*, which permits good detection of the various subtypes of HIV-1 group M and most CRFs but not of group O or HIV-2 (Triques et al., 1999; Katsoulidou et al., 2006; Schumacher et al., 2007). Abbott's tests (LCx HIV RNA quantitative, real-time HIV-1 assay for use on the m2000 system) amplify a conserved sequence in the integrase region of *pol* and detect groups N and O in addition to the various subtypes and CRFs of group M (Swanson et al., 2005; Swanson et al., 2006; Swanson et al., 2007). Although the intended primary purpose of these tests is for patient monitoring (virus load determination), they are used also diagnostically, particularly for diagnosing acute HIV-1 infection.

NASBA and TMA. In the NASBA and TMA procedures, RNA is amplified in an isothermal multienzymatic procedure mediated by the enzymatic effects of RNA-dependent DNA polymerase (RT), RNase H, DNA-dependent DNA polymerase, and DNA-dependent RNA polymerase. This procedure thus mimics the retroviral nucleic acid replication cycle (Fig. 1). The product of this amplification is a single-stranded RNA (Kievits et al., 1991). In current NASBA and TMA kits, the resulting RNA product is detected by a specific DNA probe (molecular beacon) which, when annealed to the target RNA, forms a stem-loop structure, resulting in emission of a chemoluminescent signal.

Currently available kits include a CE-marked and FDA-approved quantitative real-time NASBA kit (NucliSENS EasyQ) from bioMerieux, Marcy l'Etoile, France, which amplifies a sequence in the *gag* gene, and a TMA-based HIV-1 RNA qualitative assay (Aptima HIV-1 RNA qualitative assay) manufactured by Gen-Probe, San Diego, CA. The latter is an FDA-approved assay for diagnosis of HIV-1 infection in human plasma. The assay amplifies conserved sequences in both the LTR and *pol* of HIV-1, thus enabling simultaneous detection of all known groups of HIV-1, i.e., M, N, and O. It is intended for use in the diagnosis of HIV-1 infection, including acute or primary infection (Roland et al., 2004). The same test principle and target sequences also are used in an FDA-approved test for fully automated blood donor screening with simultaneous detection of HIV-1, hepatitis B virus, and hepatitis C virus RNA (Linnen et al., 2002; Candotti et al., 2003; Koppelman et al., 2005).

bDNA. In the bDNA method, viral RNA is captured on a solid surface by immobilized specific capture probes. The captured RNA is then reacted with "connector" probes, which with one end hybridize to a series of short sequences of the *pol* region of the RNA and with the other end mediate fixation of bDNA detector probes. These bDNAs are then reacted with still other bDNAs that hybridize to the first. Enzyme-labeled tracer probes are finally hybridized to all the branches, and the analysis is based on chemoluminescence (Urdea et al., 1993). The principal difference of the bDNA method compared with PCR or NASBA-TMA is the lack of amplification of viral sequences and, in consequence, lack of a carryover problem. What is called signal amplification is in fact a mere signal accumulation in which interacting molecular probes added to the reaction mixture at predetermined high concentrations are hybridized to captured viral RNA in an ordered process which results in the deposition of consecutive probe layers. The procedure is in fact comparable to an indirect antibody-binding assay. Higher precision is probably achieved than with nucleic acid amplification techniques, whose outcome depends on the efficacy of primer annealing in each cycle.

A bDNA-based assay for HIV-1, the Versant HIV-1 RNA 3.0 (bDNA), is sold by Bayer HealthCare, Leverkusen, Germany (Galli et al., 2005). This test is based on about 40 different probes that cover most of the *pol* gene and permit detection of the various subtypes of group M but not of group O. A drawback of the method is the relatively high detection limit, which must be compensated for by large specimen volumes (2 ml of plasma).

Nucleic acid tests with FDA approval and their intended uses are listed at http://www.fda.gov/cber/products/testkits.htm. With the exception of TMA and the RT-PCR-based systems for blood donor screening, the kits described above are designated primarily for quantification of viral RNA. Depending on the specificity and level of detection, some of them under certain conditions also may be suitable for diagnostic (qualitative) purposes, but caution must be exercised, since false-positive results have been reported (Roland et al., 2004). Of note, the cut-off for the Bayer Versant HIV-1 RNA 3.0 (bDNA) has been set in a way that will result in 5% false-positive results when used on uninfected controls! For diagnostic questions, it is therefore safer to use qualitative tests such as the Aptima HIV-1 RNA qualitative assay (Gen-Probe). There is one CE-marked commercial PCR kit for HIV-1 DNA (Roche), but there are reports of false-negative results with this kit, particularly with non-B subtypes (Bogh et al., 2001; Obaro et al., 2005). Many diagnostic laboratories, including ours, have developed their own PCR methods for qualitative detection of viral DNA or RNA. Detailed step-by-step instructions for our diagnostic procedures, which are capable of detecting a single DNA copy even without using nested PCR protocols (which carries a high risk of carryover contamination), were published previously (Boni, 1996). For quantification of viral RNA, a number of excellent real-time RT-PCR systems have been described (Drosten et al., 2006; Muller et al., 2007; Rouet et al., 2007).

The availability of commercial kits for the detection of HIV-1 RNA or DNA by sequence or signal amplification has rendered these tests attractive for laboratories with no background in molecular biology. The fact that under optimal circumstances a single gene copy can be detected by some of these tests has created a relatively uncritical attitude, resulting in a degree of trust that is not justified in several aspects. Molecular tests are sensitive to sequence variation, resulting in underdetection or false-negative results in some cases. Carryover contamination, whose main source is amplified DNA but which may also originate from other specimens, can also be a problem, and commercial products or systems must therefore have built-in carryover protection devices. In addition, precautionary measures are vital for all laboratories performing such tests (Boni, 1996).

Important factors in molecular testing are sample handling and sample preparation. Particle-associated RNA in plasma has been claimed to be very unstable, demanding special expensive plasma preparation tubes from a certain manufacturer and immediate separation of plasma from the cellular pellet before the sample is shipped to the laboratory. Independent investigators have not been able to confirm these claims. Their work indicates that HIV-1 RNA levels are stable (variance, less than 0.3 log unit) for up to 3 days after collection when stored either at room temperature or at 4°C as cell-free plasma in the EDTA-plasma preparation tubes or even as EDTA-anticoagulated whole blood in regular tubes. Comparison of paired HIV-1-positive plasma and serum specimens revealed that RNA quantitation was 20 to 65% lower in serum than in plasma. EDTA-plasma is thus the preferred specimen for these assays and provides the highest levels of RNA. EDTA-plasma that is prepared and frozen within 8 h of collection can thus be trusted to be of sufficient quality for these tests. It can be thawed and frozen up to three times before the RNA levels decrease significantly (Todd et al., 1995; Ginocchio et al., 1997; Sebire et al., 1998).

The maximal sensitivity of molecular tests is limited by sample size and sequence variation. The detection limit of the PCR is a single DNA molecule. However, 1 µg of genomic DNA, which contains the DNA of approximately 150,000 cells, corresponds to the number of peripheral blood mononuclear cells (PBMC) contained in only about 75 µl of blood. Consequently, even with a detection limit of 1 provirus/µg of DNA, roughly 70,000 infected cells must be present in 5,000 ml of blood for PCR analysis to be positive. A detection limit of 10 copies per reaction is more realistic because of the Poisson distribution, which means, for example, that not every sample with a nominal concentration of 1 copy/sample indeed contains such a copy. Similarly, the sensitivity for the detection of particle-associated HIV RNA is limited by the efficiency of the reverse transcription step and by the volume of analyzed plasma. To achieve higher sensitivity ("ultrasensitive tests"), sample input has to be increased to milliliter plasma volumes (Schockmel et al., 1997). Loss of sensitivity may also be due to HIV sequence divergence. Individual mutations at a critical downstream position of an amplification primer may lead to reduced or entirely abolished amplification. Infallible tests based on sequence amplification are thus an illusion, but significant progress resulting in broader sensitivity has definitely been achieved. Subtypes A, CRF02_AE, F, and G were systematically underdetected by version 1.0 of the Roche Amplicor HIV-1 Monitor test (Coste et al., 1996; Simons et al., 1997; Debyser et al., 1998; Parekh et al., 1999). The introduction of modified primers in the subsequent version 1.5 of the test considerably improved the recognition of these clades, while group O isolates remain undetectable. Change from Monitor version 1.5 to Roche TaqMan-based RT-PCR kits may also result in significantly lower copy numbers of HIV-1 RNA in some instances.

HIV-1 subtypes A and G were insufficiently detected by earlier NASBA kits. Designation of new primers for the current real-time version of the test, NucliSens EasyQ (bioMerieux) has, however, resulted in performance similar to Roche's RT-PCR (Lam et al., 2007; Stevens et al., 2007). Underdetection of a significant fraction of subtype C samples was reported in one study from Israel (Gottesman et al., 2006) but was not seen in studies performed in South Africa (Stevens et al., 2005; Stevens et al., 2007). On the other hand, the NucliSens EasyQ, as the first test so far, has recently been shown to enable quantification of subtype A of HIV-2 (Rodes et al., 2007). Improved recognition of group O has been achieved with Abbott's RT-PCR-based tests and the Gen-Probe TMA assays.

Broad application of NAT in the diagnostic laboratory has now become feasible, as full automation of both nucleic acid extraction and test conduction has been achieved. PCR is very helpful when serology has failed to provide a clear answer. This applies in particular to specimens with borderline reactivity in screening assays or incomplete patterns on confirmatory WB or LIA, specimens from individuals with suspected primary HIV infection (although a p24 antigen test would suffice in most instances), and babies of HIV-infected mothers. For individuals with confirmed-positive HIV-1 serology, quantification of the viral RNA in plasma is essential for clinical assessment and, if positive, serves as a further confirmation of the infection.

Virus Isolation

Leukocytes are separated from anticoagulated blood by Ficoll centrifugation and cocultured with phytohemagglutinin-stimulated leukocytes from healthy blood donors. Culture supernatants are periodically assayed for p24 antigen. Cultures usually become positive within 2 weeks, but culture times of up to 60 days have been reported (Ho et al., 1989; Burgard et al., 1992). The procedure has a sensitivity of about 90% over all stages; the success rate is lower for asymptomatic patients. Significant improvement may be achieved by complicated procedures combining concentration of cells likely to be infected, removal of cells that might interfere with virus replication, such as $CD8^+$ T-cells, and activation of infected lymphocytes using phytohemagglutinin or antibody to CD3 and CD28. Such procedures enabled virus isolation from lymph node cells of all patients whose viral RNA level in plasma had become undetectable under ART (Finzi et al., 1997; Wong et al., 1997). Despite these improvements in sensitivity, virus isolation remains time-consuming and costly. Since PCR for viral DNA or RNA, as well as the signal amplification-boosted p24 antigen test, have a diagnostic sensitivity of more than 96% and yield results within 1 day, virus isolation has become unsuitable for the routine diagnosis of adult and pediatric HIV infection in both developed and resource-poor settings, although it remains important for many research questions.

RT Assays

Particle-associated RT is a unique marker of retroviruses, and RT assays have been instrumental in the discovery of all known human retroviruses. Tests have now been developed that combine the broad detection range of RT tests with the high sensitivity of nucleic acid amplification procedures. These so-called product-enhanced RT (PERT) assays are

based on the selective enhancement, by PCR or another amplification method, of the cDNA product synthesized from an RNA template by the RT activity contained in a test sample. They are 10^6 to 10^7 times more sensitive than a conventional RT test and detect fewer than 10 particles (Pyra et al., 1994), thus rivaling PCR for detecting viral RNA in plasma (Boni et al., 1996a; Reisler et al., 2001). Detailed step-by-step instructions have been published (Boni and Schupbach, 1999). Assays based on this principle were established also by others (Silver et al., 1993; Heneine et al., 1995). In the context of HIV infection, the PERT assay may be diagnostically useful when a low $CD4^+$ lymphocyte count or indeterminate serological results, such as a WB, give a result positive according to ASTPHLD-CDC but indeterminate by FDA guidelines, suggesting the presence of an HIV infection, possibly with an agent not well detected by the current HIV-specific amplification methods. A negative result with the PERT assay, which would detect any retrovirus independent of its genomic sequence, excludes infection with an aberrant HIV strain. Since a commercial kit is not available, the test is currently restricted to specialized retrovirus laboratories that also do research. A less sensitive test kit lacking amplification has been evaluated as an alternative virus load test for resource-poor settings (Seyoum et al., 2006). Unlike the PERT assay, due to the lack of sensitivity, it cannot be used for diagnostic purposes.

Diagnosis of HIV-2 Infection

Screening tests, at least in Europe, must demonstrate good sensitivity to HIV-2 to receive CE marking. Detection of HIV-2 infection at the level of screening is therefore not a problem. In contrast, determination of whether reactivity in screening is due to infection with HIV-1, HIV-2, or both is sometimes difficult. On HIV-1 WB, sera from HIV-2-infected individuals frequently have strong reactions with Gag and Pol proteins compared to their reaction with Env. In particular, they may present with an unusually strong p31 (integrase) band. A suspected HIV-2 infection is further supported by reaction with the recombinant HIV-2 TM protein present on the products of some manufacturers. We have, however, occasionally seen samples that did not react with this band, although HIV-2 infection was subsequently confirmed. The presence of isolated Gag bands on HIV-1 WB or of strong reactivity against p24 and Gag precursors (p55, p43, and p39) is not an indication of HIV-2 infection. Similarly, the presence of faint Gag-reactive bands on HIV-1 blots is not an indication for an HIV-2 WB.

Results of HIV-1 WB suggestive of HIV-2 infection may be confirmed by an HIV-2 WB or, preferably, by tests involving specific recombinant proteins, e.g., an LIA or a rapid test that differentiates between the two. If reactions to both HIV-1 and HIV-2 proteins are present at similar intensities, diagnostic PCR for proviral DNA of both viruses is necessary. HIV-2-infected asymptomatic individuals, i.e., the overwhelming majority, have much lower viral loads than those infected with HIV-1; HIV-2 RNA is usually undetectable in these patients. The confirmatory test of choice, thus, is PCR for HIV-2 DNA.

Diagnostic Algorithms and General Considerations

Guidelines for HIV testing may vary in different countries based on prevalence of virus types, available tests, laboratory facilities, health care systems, and economic or other factors. Revised U.S. recommendations for HIV testing of adults, adolescents, and pregnant women in health care settings were issued in 2006 (Branson et al., 2006); these recommendations are also accessible online (http://www.hivatis.org/Guidelines). FDA-approved test kits are listed at http://www.fda.gov/cber/products/testkits.htm.

U.S. Guidelines

Initial screening is done with an FDA-approved EIA for HIV antibodies. Specimens with a nonreactive result are considered HIV negative unless new exposure has occurred. Specimens with a reactive EIA result are retested in duplicate. If the result of either duplicate is reactive, the specimen is reported as repeatedly reactive and undergoes confirmatory testing with a supplemental test (e.g., WB or immunofluorescence assay). Specimens repeatedly reactive by EIA and positive by WB or immunofluorescence assay are considered HIV positive (Fig. 8, dark area).

Specimens that are repeatedly EIA reactive occasionally provide an indeterminate WB result, which might represent either an incomplete antibody response to HIV in specimens from infected persons or nonspecific reactions in specimens from uninfected persons. Immunofluorescent antibody can be used to resolve an indeterminate WB sample. Generally, a second specimen should be collected >1 month later and retested for persons with indeterminate WB results. Nucleic acid testing for viral RNA or proviral DNA could also help resolve an initial indeterminate WB in certain situations.

An HIV test should be considered positive only after screening and confirmatory tests are reactive. A confirmed positive test result indicates that a person has been infected with HIV. False-positive results when both screening and confirmatory tests are reactive are rare. However, the possibility of a mislabeled sample or laboratory error must be considered, especially for a person with no identifiable risk for HIV infection.

Because a negative test result probably indicates absence of HIV infection, a negative test need not be repeated in persons with no new exposure in settings with low HIV prevalence. For persons with a recent history of known or possible exposure to HIV who are tested before they could develop detectable antibodies, the possibility of HIV infection cannot be excluded without follow-up testing. A false-negative result also should be considered in persons with a negative HIV-1 test who have clinical symptoms suggesting HIV-1 infection or AIDS. Additional testing for HIV-2 and HIV-1 group O infection might be appropriate for these persons.

Most people with an initial indeterminate WB result who are infected with HIV-1 will develop detectable HIV antibody within 1 month. Thus, persons with an initial indeterminate WB result should be retested for HIV-1 infection >1 month later. Individuals with continued indeterminate WB results after 1 month are unlikely to be HIV infected and should be counseled as though they are not infected unless recent HIV exposure is suspected. Nucleic acid tests for HIV DNA or RNA are not generally recommended for resolving indeterminate WB results except in suspected cases of primary or acute infection (Divine et al., 2001).

Alternative Testing Strategies

In contrast to the United States, where screening does not normally include testing for HIV-2 or group O viruses, CE-marked screening tests used in Europe must detect all HIVs. Great importance is placed also on the detection of primary HIV infection; therefore, fourth-generation screening tests or additional antigen testing are recommended whenever a primary HIV infection must be considered. Finally, verification of all confirmed HIV-positive results with a second,

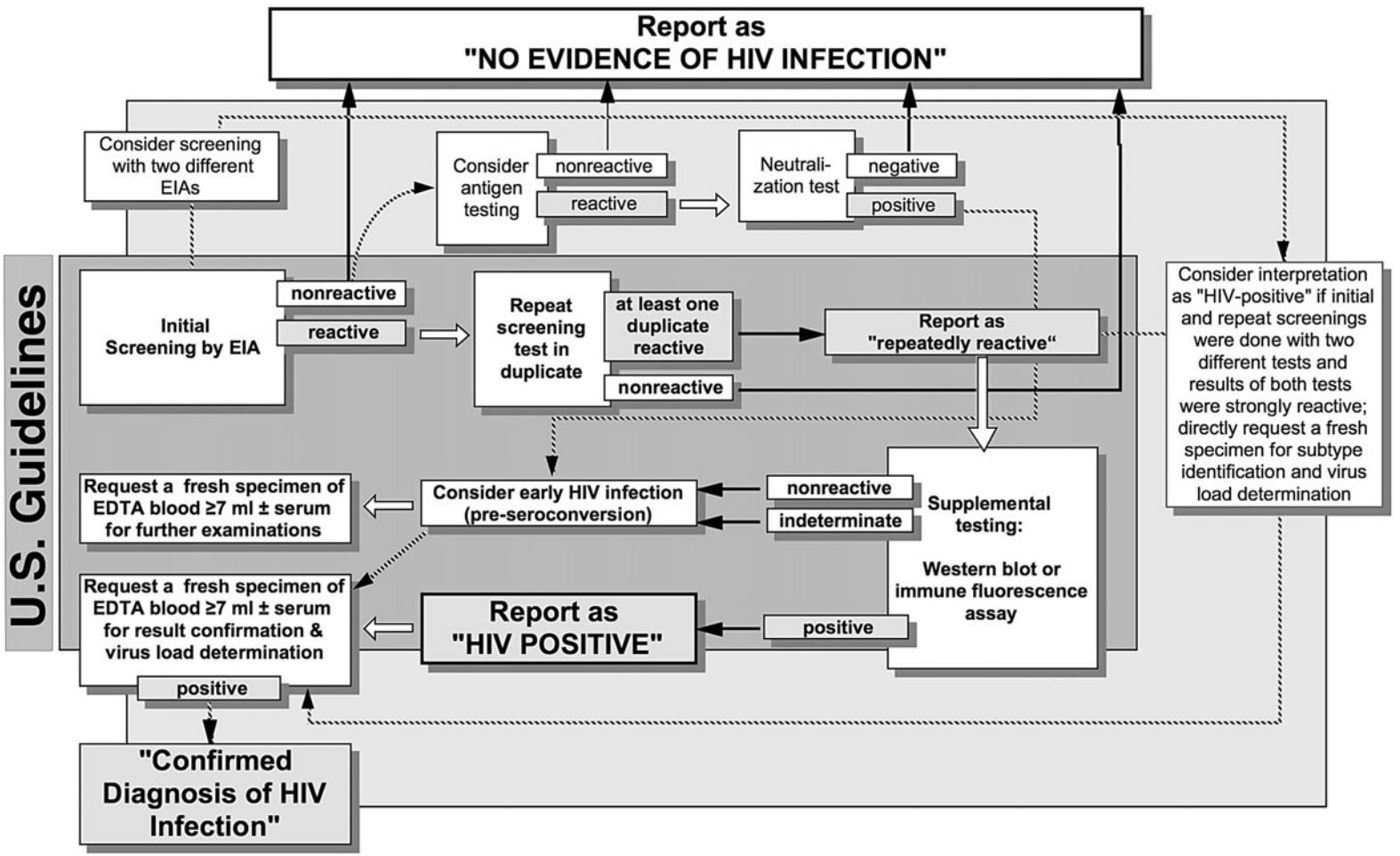


FIGURE 8 Algorithms for diagnosis of HIV infection in adults. The darker shading represents algorithms of U.S. guidelines; the lighter shading illustrates possible alternatives. Reprinted from Schüpbach, 2003b, with permission.

freshly drawn specimen is mandatory when a diagnosis of HIV infection is first established. The peripheral, lightly shaded areas of Fig. 8 illustrate possible modifications of the U.S. recommendations that may under certain circumstances be useful.

To avoid the practical and financial problems associated with WB testing, the WHO has recommended alternative test strategies based on the use of at least two different screening tests (Sato et al., 1994). Large studies have shown that such testing algorithms may yield results that are at least equivalent to the conventional testing algorithm outlined above. In one alternative algorithm, reactive samples are subjected to a different screening assay, and only those samples with discrepant results are subjected to WB testing. Initial screening may also be performed with two different tests, and those with discrepancies undergo supplemental testing. Supplemental testing by WB can also be replaced without loss in sensitivity or specificity by a third screening assay (Laleman et al., 1991; van der Groen et al., 1991; Urassa et al., 1992; Nkengasong et al., 1999). Given the further improvement of screening tests since the time most of these studies were done, such testing algorithms have become even more attractive. In particular, initial screening with a fourth-generation antibody or antigen test and a third-generation DAGS test in parallel is bound to increase the sensitivity. Samples nonreactive in both assays are reported as HIV negative. Samples reactive in both assays are almost always infected, since the high diagnostic specificity (>99.5%) of each of the two tests is potentiated, resulting in an overall diagnostic specificity of ≥99.9975%. Therefore, a freshly drawn confirmatory sample can be directly used for quantification of the viral RNA load. An HIV-1 RNA concentration above about 5,000 copies/ml plasma is sufficient additional proof of an infection but is not specific for HIV-1. As some HIV-2 isolates are recognized relatively well by current RT-PCR test kits while others are severely underdetected or recognized not at all, there is always the possibility that a patient with a low to medium level of detectable RNA in an HIV-1 test is actually infected with HIV-2 (Schutten et al., 2004). Serological tests able to distinguish between HIV-1 and HIV-2 are thus the best solution to diagnose, or exclude, HIV-2 infection.

Result discrepancy between the two screening tests or borderline results may be due to acute HIV infection or a nonspecific reaction. Acute infection must be suspected if reactivity is restricted to the fourth-generation assay; a reactive antigen test confirmed by a positive antigen neutralization test further supports this possibility. If the antigen test result is negative, seroconversion is unlikely, and false reactivity must be suspected. Since inconsistent or indeterminate results may be due to laboratory errors (sample mix-up, carryover contamination), diagnostic clarity is sometimes best achieved with a freshly drawn sample. An EDTA-anticoagulated blood sample of at least 7 ml should be requested. This will permit performance of any of the supplemental tests. Since NAT almost always become positive prior to seroconversion, there is no rationale for a prolonged interval to clarify indeterminate antibody test results. If PCR for viral RNA or DNA is negative in such samples, an HIV infection as the reason for the indeterminate antibody result can be excluded for practical purposes.

Assays performed on the second specimen are chosen according to the results of the first sample. A confirmatory laboratory should be capable of performing a variety of supplemental tests to establish a "confirmed diagnosis of HIV infection." A third positive screening assay, a positive WB according to FDA guidelines, a positive and neutralized antigen assay, or PCR for viral RNA or DNA may be used alone or in combination to establish such a diagnosis, with the results obtained with the first sample also being taken into consideration (case interpretation). Tests performed with the second sample should also establish the type of virus (HIV-1 or HIV-2) and the viral RNA load and may, if indicated, even include antiretroviral resistance testing.

It is important that the diagnosis of an HIV infection never be established on a single specimen. The possibility that an error might lead to a false-positive diagnosis dictates verification of all reactive results with a second, freshly drawn sample. Also, indeterminate results of different methods never add up to a positive result. For example, a borderline screening test plus a borderline WB with a pattern ruled positive according to guidelines issued by the ASTPHLD-CDC but indeterminate by ARC or FDA criteria, plus a reactive antigen test which, however, cannot be confirmed by neutralization are not sufficient for a confirmed positive diagnosis. Testing must continue until clear-cut positive results are obtained.

Diagnosis of Pediatric HIV-1 Infection

Diagnosis of HIV infection in babies born to HIV-positive mothers is complicated by the presence of HIV-specific IgG antibodies of maternal origin; HIV IgG concentrations in term-born babies are as high as in their mothers. Since the half-life of IgG is about 3 weeks, HIV-specific maternal antibodies disappear slowly and may remain detectable for up to 15 to 18 months. Early diagnosis of HIV infection in maternally exposed infants is thus only possible with tests for virus components. PCR for proviral DNA in blood cells or HIV-1 RNA in plasma have become the methods of choice (Nielsen and Bryson, 2000). Approximately one-third of maternally transmitted infections, probably those representing transmission in utero, can be detected within the first 10 days of life. In a few cases, PCR for DNA from PBMC may still be negative when PCR for viral RNA in plasma is already positive (Steketee et al., 1997; Cunningham et al., 1999; Young et al., 2000). The remainder, assumed to have become infected at birth, become PCR positive within the next 2 months. Several studies have shown that testing of heat-denatured plasma samples by signal amplification-boosted p24 antigen EIA diagnoses pediatric HIV-1 infection with sensitivity and specificity similar to that of tests for viral DNA or RNA (Nadal et al., 1999; Fiscus et al., 2006). Tests for HIV-1 DNA, RNA, or p24 antigen can also be conducted on dried blood spot specimens (Patton et al., 2006; Knuchel et al., 2007; Patton et al., 2007).

HIV Disease and Treatment Monitoring

Determination of the HIV RNA concentration (viral load) is instrumental in several aspects for the clinical management of HIV infection (for updated online comprehensive treatment information and guidelines, refer to http://aidsinfo.nih.gov/ or http://www.hivatis.org/?list/). First, in early infection at the set point it serves to assess the likely course the infection will take. In untreated patients, the viral load and $CD4^+$-T-cell count are measured every 3 to 4 months. Treatment decisions are taken based on the $CD4^+$-T-cell count and the viral load. When patients start ART or after a change in ART, drug efficacy is initially assessed by measuring the viral load after 2 to 8 weeks. The next viral load measurement for assessment of antiviral effect is performed 3 to 4 months after start of ART, and at 3- to 4-month intervals thereafter. Outside of such regular measurements,

the viral load is also determined in case of clinical events or a significant decline in CD4+ T cells (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2006).

Prognostic Value of Viral Load

Higher HIV RNA levels correlate with lower baseline CD4+-T-cell counts, a more rapid decline in CD4+-T-cell counts, and more rapid disease progression. Patients with more than 100,000 copies/ml of plasma within 6 months of seroconversion were 10 times more likely to progress to AIDS over 5 years than were those with fewer copies. Maintenance of <10,000 copies/ml in early HIV infection is associated with a decreased risk of progression to AIDS. In contrast, in patients with more advanced disease, a low RNA count does not protect from progression; up to 30% of patients with <10,000 copies/ml progressed (Coombs et al., 1996; Mellors et al., 1996; Saag et al., 1996; Welles et al., 1996; O'Brien et al., 1997). Patients with advanced disease can present with high or low viral RNA concentrations (Saag et al., 1996).

RT-PCR, NASBA, and bDNA methods are valid procedures for viral load quantification, and the FDA-approved respective kits include the Amplicor HIV-1 Monitor test version 1.5 (Roche Diagnostic) with a detection limit of 50 copies/ml; the NucliSens HIV-1 QT (bioMerieux) with a detection limit of 80 copies/ml, and the Versant HIV-1 RNA 3.0 assay (Bayer) with a detection limit of 75 copies/ml. The minimal change in viral load considered to be statistically significant (2 standard deviations) is a threefold or 0.5 $\log_{10}$ copies/ml change. In the plasma of most untreated patients, viral RNA is detectable at all stages of disease. If RNA is undetectable in an untreated patient, this may reflect a very low viral load, as seen in long-term nonprogressors. However, the negative result may also be due to a virus not well recognized by the respective assay. The above-mentioned kits are not FDA approved for measurement of HIV-1 group O or HIV-2. Although underdetection of entire subtypes or clades by some tests meanwhile has been much improved, the current virus load kits may still exhibit weaknesses in detection of certain CRFs or individual virus strains (Gueudin et al., 2007). This may also extend to subtype B viruses (Schüpbach, unpublished).

As a general rule, if quantitative tests of untreated patients yield an undetectable viral load, one should check the likelihood of an infection with an alternative subtype. This is particularly important in case of a low CD4+-T-cell count or clinical events. A negative result in other virus component tests less affected by sequence variation supports a truly low load. Such alternative methods might also include the PERT assay, which, as a functional test for RT activity, is entirely independent of viral sequence and can therefore be used for quantification of HIV-1 subtype O or HIV-2 (Bürgisser et al., 2000). Contrary to general perception, p24 antigen is also a valid prognostic marker when assessed by optimized procedures (Ledergerber et al., 2000; Sterling et al., 2002). Short-term changes in CD4+-T-cell counts in both adult and pediatric HIV-1 infection correlated even better with the corresponding changes in concentration of HIV-1 p24 than with those of HIV-1 RNA (Schupbach et al., 2005; Brinkhof et al., 2006).

ART and Its Monitoring

Eradication of HIV infection cannot be achieved with available antiretroviral regimens. This is due to the pool of latently infected CD4+ T cells that is established during early HIV infection and persists with a long half-life. Hence, the primary goals of therapy in HIV-infected patients are to reduce HIV-related morbidity and mortality, to improve quality of life, to restore and preserve immunologic function, and to maximally and durably suppress viral load (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2006). Treatment with effective combinations of antiretroviral drugs has resulted in substantial reductions in HIV-related morbidity and mortality. Plasma viremia is a strong prognostic indicator of HIV disease progression. Reductions in plasma viremia achieved with such combination ART account for substantial clinical benefits (O'Brien et al., 1996). Therefore, suppression of plasma viremia as much as possible for as long as possible is a critical goal of ART.

Due to the high variability of HIV and the generation of drug-resistant mutants, successful long-lasting suppression of virus replication can only be achieved with combination ART, frequently also called highly active ART. Such regimens are usually composed of at least three drugs selected from the groups of NNRTI, protease inhibitors (PIs), and nucleoside (or nucleotide) analog RT inhibitors (NRTI). NRTIs function as nucleoside triphosphates for RT-mediated cDNA synthesis and act as chain terminators. NNRTI bind directly to the RT, thereby blocking its active site either directly or indirectly. PIs block the active site of the viral PR, thereby inhibiting the processing of the Gag-Pol and Gag precursor proteins. More recently, entry and fusion inhibitors have become available, which block fusion-mediated virus entry into host cells. Novel drug classes now in clinical use include integrase inhibitors and CCR5 coreceptor antagonists, while CXCR4 antagonists are still under clinical evaluation. For FDA-approved or investigational drugs and all questions regarding ART, consult http://aidsinfo.nih.gov/.

Treatment monitoring assesses the treatment-induced reductions of the amounts of virus in the body. Effective ART decreases HIV-1 RNA concentrations in plasma by at least 1 $\log_{10}$ within 8 weeks after start of treatment, to less than 400 copies/ml by 24 weeks and to less than 50 copies/ml by 48 weeks. A virologic failure of treatment is present if these cornerstones are not met or if there is a repeated HIV RNA level above 400 copies/ml after prior suppression of viremia to lower than 400 copies/ml (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2006).

The decrease in HIV RNA concentration in plasma is accompanied by a slow decrease in PBMC-associated proviral DNA. Analysis of lymphoid tissues shows, however, that virus-expressing cells, not to mention provirus, are still detectable after years, and replication-competent virus can also be isolated from lymphoid tissues of such patients (Finzi et al., 1997; Wong et al., 1997; Finzi et al., 1999). Interruption of the treatment regimen for brief periods may thus, within a few days, lead to reappearance of virus in plasma to levels seen prior to therapy (Neumann et al., 1999; Fischer et al., 2003).

Concentrations of viral RNA in plasma are thus an imperfect reflection of the HIV situation in the lymphatics, which harbor 98% of the body's lymphocytes. The rapid decline of viral RNA levels in plasma of patients receiving highly active ART is nevertheless paralleled by a similar decline of viral RNA levels in the lymphatics (Cavert et al., 1997) and the GALT (Guadalupe et al., 2006; Poles et al., 2006). In tonsil tissue, there was a rapid drop in mononuclear cells acutely producing virus, with a half-life of 0.9 day, which is comparable to the 1.1 days observed for acutely infected CD4+ T cells in the blood under similar treatment conditions. Viral RNA levels at the surface of follicular DC declined with an initial half-life of 1.7 days followed by a

slower decay with a half-life of 14 days. Cell-associated viral RNA levels declined with a similar half-life. After 6 months, there were still infected cells and low levels of virus expression in the majority of patients (Cavert et al., 1997); cell-associated viral DNA and mRNA levels reached a plateau after about 500 days of treatment, after which no further decrease was observed (Furtado et al., 1999). Ongoing low-level replication after more than 18 months of aggressive treatment has been confirmed by the demonstration of unintegrated circular forms of viral DNA (Fig. 1C) or of sequence evolution (Chun et al., 1997; Furtado et al., 1999; Zhang et al., 1999).

Viral load studies of patients receiving ART have so far focused on effects in plasma and the lymphatics. Little is known about the impact of these regimens on HIV infection of the CNS, and viral kinetics in the CNS have not been established.

RNA-based tests are currently viewed as the only feasible methods of viral load determination, and all treatment recommendations are based on HIV-1 RNA. Treatment monitoring based on real-time PERT assay, though not available commercially, would also be feasible, however, and is an excellent alternative to sequence-based tests (Bürgisser et al., 2000). In addition, the measurement of p24 antigen represents a valuable simple alternative for resource-poor settings. In both adult and pediatric patients, HIV RNA and p24 behave similarly, in certain cases virtually identically (Boni et al., 1997; Nadal et al., 1999; Tehe et al., 2006). Similar to early PCR kits (Monitor HIV-1 RNA version 1) there is, however, underdetection of certain non-B subtypes, particularly subtype D, while the sensitivity to subtype C is good (Knuchel et al., 2007).

HIV Drug Resistance Testing

The Achilles heel of any antimicrobial chemotherapy is the development of resistance. Antiretroviral resistance develops when viral replication continues in the presence of the selective pressure of drug exposure. In the case of the antiretroviral drugs used against HIV, this is caused most frequently by mutations of the genes targeted by these drugs, namely, the genes coding for RT, PR, IN, or gp41. Mutations conferring resistance may, however, also occur outside the protein sequences targeted by antiretroviral drugs, as shown by certain mutations in the proteolytic processing sites encoded by the *gag* gene, which are targeted by the viral PR (Carrillo et al., 1998). Since many resistance mutations affect different drugs, resulting in cross-resistance, a detailed knowledge of these mutations is valuable for the design of treatment in individual patients. This is particularly true in view of the fact that viruses with resistance mutations may be transmitted and thus may be present in patients prior to any ART. The frequency of such transmissions in the United States and Western Europe is estimated at 10 to 15%; transmission of multidrug-resistant viruses also has been observed (Weinstock et al., 2004; Descamps et al., 2005; Novak et al., 2005; Oette et al., 2006).

There are two types of resistance mutations, primary and secondary. Primary mutations directly reduce the susceptibility of the virus to an antiretroviral drug, are relatively specific for each drug, and appear soon after treatment initiation. They permit the mutated virus to replicate in the presence of the drug, but its replicative capacity is usually impaired due to a decreased functional efficiency of the mutated enzyme or protein. When treatment with the failing drug is continued, virus strains with secondary mutations, which compensate for the impairment, will be selected over time. Most of these secondary (or compensatory) mutations do not further increase resistance to the drug but restore the replicative capacity of the mutant virus. While there is little overlap among primary mutations, many of the compensatory mutations are shared among drugs of the same family, NRTIs, NNRTIs, and PIs. Failing drug regimens should therefore be switched as soon as they are recognized, to prevent secondary mutations (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2006).

Not all apparent resistance to ART is due to viral mutation and drug-induced selection. Viruses of group O and HIV-2 are intrinsically resistant to NNRTIs, since their RTs do not bind these drugs, which were developed based on the B subtype prevalent in industrialized countries (Parkin and Schapiro, 2004). For some drugs, such as stavudine, cellular phosphokinases are needed to activate the drug to its triphosphate form intracellularly; previous treatment with zidovudine appears to decrease this enzyme activity, thus impairing the intracellular concentrations of active stavudine. Frequently, however, the reasons for a failing viral response to ART are a lack of adherence to treatment, impaired intestinal drug absorbance, pharmacokinetic interactions, or continuing viral replication at sanctuary sites where drug concentrations are inadequate. These points should be evaluated before a resistance analysis is considered (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2006).

Current guidelines issued in 2003 by the International AIDS Society-USA (IAS-USA) panel and in 2004 by a European panel recommend drug resistance testing in the setting in cases of acute or recent HIV infection, for patients who have been infected as long as 2 years or more prior to initiating therapy, in cases of antiretroviral failure, and during pregnancy (Hirsch et al., 2003; Vandamme et al., 2004).

The methods include genotypic and phenotypic assays (reviewed in Hirsch et al., 2003). Genotypic tests examine the population of viral genomes in a test sample for the presence of mutations known to confer resistance. More than 100 resistance-associated mutations have been described. An updated list of HIV-1 resistance mutations is maintained by the IAS-USA Drug Resistance Mutations Group (Johnson et al., 2006) and also accessible online (http://www.iasusa.org/resistance_mutations/). Phenotypic assays measure the degree of drug sensitivity of a population of viruses or the enzyme(s) targeted by the drug; the result of this assay is given as a 50 or 90% inhibitory concentration. Genotypic assays use RT-PCR to amplify the population of viral RNA sequences which code for the viral enzymes RT and PR, which are targeted by most of the currently available antiretroviral drugs. The analysis of the amplified sequences for known resistance mutations can then be done by various methods. One method uses reverse hybridization of the amplified material to sequence probes that are immobilized on a carrier surface. This principle is also used in the line probe assay, in which a limited number of mutations (a limitation which renders the test unsuitable for clinical practice) (Garcia-Bujalance et al., 2005) are assessed by hybridizing the amplified sequence to probes that discriminate between wild-type and mutant sequences and are immobilized on a WB-like strip. On a more general scale, this principle is also utilized in chip-based high-density oligonucleotide arrays. Another frequently used method of product analysis is by automated sequence analysis of the target region.

Phenotypic assays are performed with viruses isolated from patients and grown to sufficient quantity in a suitable target cell culture. To improve standardization, phenotypic

resistance analysis also is performed by a recombinant-virus approach. In this method, the respective target gene sequence is reverse transcribed from RNA that has been extracted from the patient's plasma, amplified, and inserted into a cloned defective virus backbone that lacks the sequences of interest. The reinsertion of these sequences renders the virus again competent for replication in a given cell line, and the recombinant viruses will exhibit standardized replication properties for all gene products except the inserted target gene sequences, which represent the patient's own virus population.

A problem of all resistance assays is their limited capability to detect a minority of resistant mutants against a background of wild-type sequences. Even under optimal conditions, resistant sequences usually need to be present in at least 25 to 30% of the circulating virions to be reliably detected. Under practical conditions, as assessed in a round trial, in which panels with standardized mixtures of wild-type and mutant sequences of a cloned virus were assessed by genotyping laboratories around the world, results were far from optimal, indicating as in the early days of PCR, the urgent need for standardization of these procedures and quality control (Schuurman et al., 1999). With these measures in place, there is now both retrospective and prospective evidence in support of both clinical utility of resistance testing, especially when combined with expert interpretation (Deeks et al., 1999; Baxter et al., 2000; Clevenbergh et al., 2002; Torre and Tambini, 2002; Ena et al., 2006) and cost-efficiency (Sax et al., 2005; Sendi et al., 2007).

HTLV-1, -2, -3, AND -4

Biology

PTLV

The HTLV are members of the group of PTLV. Together with bovine leukemia virus, the PTLV form the genus *Deltaretrovirus* in the *Orthoretrovirinae* subfamily of the *Retroviridae*.

The first PTLVs identified were the human T-lymphotropic viruses HTLV-1, discovered independently in the United States and Japan (Poiesz et al., 1981; Yoshida et al., 1982), and HTLV-2 (Kalyanaraman et al., 1982). Subsequent investigations among nonhuman primates demonstrated related viruses in many different species of Old World monkeys. Phylogenetic analysis separated the PTLV into three different branches, PTLV-1, -2, and -3. Depending on whether PTLV are found in humans or nonhuman primates, they are now named either HTLV-1, HTLV-2, STLV-1, STLV-2, or STLV-3. Hitherto unknown human viruses related to STLV-3 were recently identified in African hunters from Cameroon (HTLV-3), and a new human virus forming a fourth branch of PTLV, HTLV-4, was also identified (Calattini et al., 2005; Wolfe et al., 2005; Calattini et al., 2006; Switzer et al., 2006). Figure 9 summarizes the phylogenetic relationship of the PTLV. It documents that HTLV infection in humans has resulted from multiple cross-species transmissions of STLV in the past. Such zoonotic transmission may well be ongoing.

The finding of HTLVs in now four distinct clades suggests an ancient evolution. Molecular studies estimate the PTLV ancestor to have originated about 630,000 to 950,000 years ago, confirming an ancient evolution of primate deltaretroviruses (Salemi et al., 2000; Switzer et al., 2006). The separation of PTLV-1 and PTLV-2 occurred between 580,000 and 870,000 years ago, while HTLV-2 and STLV-2 diverged around 190,000 to 290,000 years ago. The PTLV-3 progenitor was estimated to have appeared between 63,000 and 95,000 years ago, with the ancestor of HTLV-3 arising about 36,087 to 54,067 years ago (Switzer et al., 2006).

HTLV-1 comprises different subtypes A to F. The cosmopolitan subtype A includes the prototype isolates from Japan and is found in many areas of endemicity worldwide. Its current worldwide distribution is thought to result from relatively recent human migration, such as the European voyages of discovery of past centuries and the slave trade. Subtypes B, D, and F are still restricted to Central Africa. Subtype E is prevalent in South and Central Africa, and subtype C is found in Melanesia (Seiki et al., 1982; Sherman et al., 1992; Gessain, 1996; Slattery et al., 1999).

HTLV-2 comprises two main subtypes, A and B (Hall et al., 1992). Both are present in intravenous drug users in North America, Europe, and Asia and have been found sporadically in Africa. HTLV-2a is present in certain American Indian tribes of North, Central, and South America, including the Navajo and Pueblo in New Mexico and the Kayapo, Kraho, and Kaxuyana in Brazil. A subcluster of Brazilian Indian HTLV-2a strains has been proposed to represent a different subtype HTLV-2c (Eiraku et al., 1996). Due to a high prevalence in isolated Amerindian populations, HTLV-2 was originally considered to be of New World origin. The discovery of endemic HTLV-2 infections in remote Pygmy populations and the identification of a simian virus closely related to HTLV-2 in bonobos suggest, however, that HTLV-2 rather has its origin in Africa. The molecular characterization of an HTLV-2b isolate from a Cameroonian Pygmy and a Congolese Efe Pygmy HTLV-2 strain belonging to a potential new subtype, HTLV-2d, also support an ancient African origin of HTLV-2 (Vandamme et al., 1998).

Genome, Transcripts, and Viral Proteins

The HTLV genome contains, downstream of the *gag*, *pol*, and *env* genes, an additional coding region called pX (Fig. 10). The pX region codes for regulatory and accessory genes in four ORFs (pX ORFs I to IV). The regulatory proteins encoded by pX ORFs III and IV include p40-Tax and p27-Rex. These proteins have been characterized extensively.

Tax (40 kDa in HTLV-1 and 37 kDa in HTLV-2, respectively) is a potent *trans*-activator of HTLV expression (Kiyokawa et al., 1984; Sodroski et al., 1984). Tax does not bind to the LTR directly but activates transcription by recruiting, or modifying the activity of, cellular transcription factors including cyclic AMP-responsive element binding protein, serum-responsive factor, and NF-κB. Three highly conserved 21-bp repeat elements located within the LTR, commonly referred to as Tax-responsive element 1, are critical to Tax-mediated viral transcriptional activation through complex interactions with cellular transcription factors (Grassmann et al., 2005). Tax also has been shown to activate transcription from a large number of critical cellular genes through the NF-κB and serum-responsive factor pathways (reviewed in Grassmann et al., 2005; Hall and Fujii, 2005; Kashanchi and Brady, 2005; Sun and Yamaoka, 2005).

Rex (p27 for HTLV-1; p26 for HTLV-2) is a splicing suppressor of the viral transcripts functionally similar to HIV Rev (Seiki et al., 1985). It recognizes a specific response element on incompletely spliced viral mRNAs, stabilizes them, inhibits their splicing, and transports them to the cytoplasm. Rex is indispensable for efficient viral replication, infection, and spread. It is considered to regulate the switch between latent and productive HTLV infection. Without Rex, the virus would still produce regulatory and some accessory proteins;

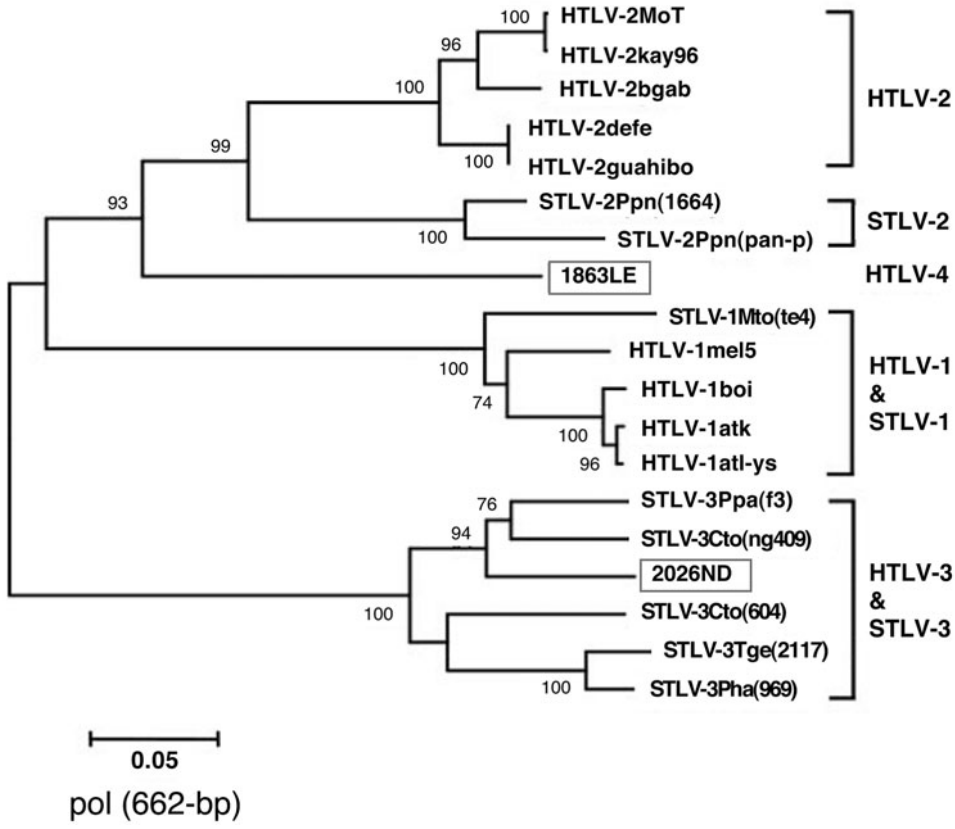


FIGURE 9 Phylogenetic relationships of PTLV based on polymerase (*pol*; 662 bp). Sequences isolated from humans in the study by Wolfe et al. (2005) are shown in boxes. Support for the branching order is based on 1,000 bootstrap replicates; only values of 60% or more are shown. Branch lengths are proportional to the evolutionary distance (scale bar) between the taxa. Taxa abbreviations are: Ppn, *Pan paniscus* (bonobo); Mto, *Macaca tonkeana* (Celebes macaque); Ppa, *Papio papio* (Guinea baboon); Cto, *Cercocebus torquatus* (red-capped mangabey); Tge, *Theropitecus gelada* (gelada baboon); Pha, *Papio hamadryas* (sacred baboon). Reprinted from Wolfe et al., 2005, with permission.

however, structural and enzymatic posttranscriptional gene expression would be severely repressed, essentially leading to a nonproductive state of infection (reviewed by Younis and Green, 2005).

The interplay of Tax and Rex leads to a sequential and, to some degree, transitory viral expression in infected cells (Seiki et al., 1988; Yoshida et al., 1989). As summarized in Fig. 11, an initial HTLV-1 transcript is fully spliced into a pX mRNA encoding for p40-Tax and p27-Rex. Tax *trans*-activates the transcription of the viral genome; thus viral expression is potently enhanced. Next, p27-Rex, which is encoded by the same pX mRNA species, accumulates and suppresses the splicing of the viral transcripts. As a consequence, unspliced *gag-pol-env* and singly spliced *env* mRNAs are expressed and viral structural proteins produced, while at the same time, the level of fully spliced pX mRNA encoding for Tax decreases, thus resulting in downregulation of viral gene expression.

Tax is a tumorigenic protein, as is shown by the induction of mesenchymal tumors in transgenic mice (Nerenberg et al., 1987). This finding implies that Tax is not only a potent activator for HTLV's own transcription but must also act on cellular genes. Numerous investigations indeed have demonstrated that Tax influences the expression of many different cellular genes, and the initial steps in the pathogenesis of ATLL are firmly linked to the pleiotropic activity of Tax. In brief, and as described in detail in recent reviews (Neuveut and Jeang, 2002; Franchini et al., 2003; Jeang et al., 2004; Kehn et al., 2004; Kashanchi and Brady, 2005; Pise-Masison and Brady, 2005; Yoshida, 2005), Tax induces abnormal cell growth by activating growth-promoting genes, by repressing growth suppressing genes, and by inhibiting tumor suppressor proteins. During abnormal cell proliferation, once thus induced, Tax also suppresses DNA repair capacity and bypasses the cell cycle checkpoint by inactivation of checkpoint function, thereby enhancing accumulation of mutations. Furthermore, Tax inhibits apoptotic cell death even in cells with abnormally damaged DNA. Over repeated cell cycles, some cells may fortuitously accumulate a combination of DNA mutations that trigger transformation and progress into malignant conversion. The pleiotropic effects of Tax are thus comparable to the many steps required for tumorigenesis in other cancers, which occur sequentially over time and at a low rate of incidence (Yoshida, 2005).

In contrast to Tax and Rex, the contribution of the four accessory proteins p12(I), p27(I), p13(II), and p30(II) to viral replication and pathogenesis is still rather unclear. Although they are dispensable for replication in vitro, the

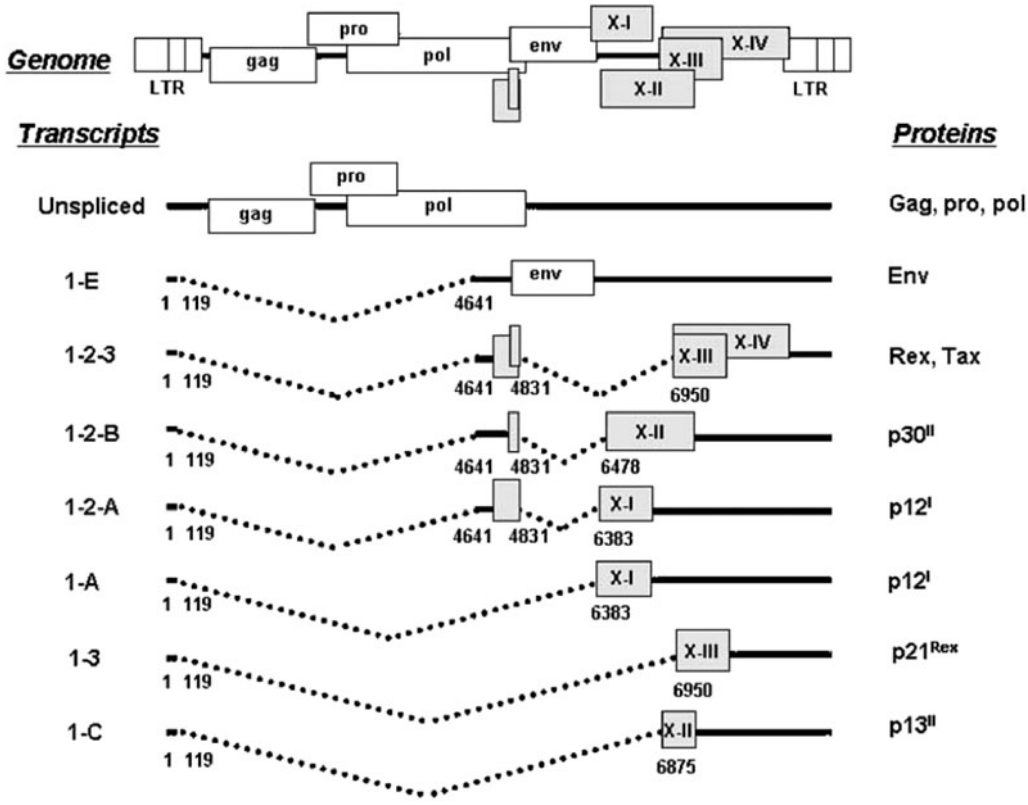


FIGURE 10 HTLV-1 ORFs and transcription map. A scheme of the HTLV-1 genome, alternatively spliced mRNAs, and putative proteins encoded by each mRNA is shown. ORFs are indicated by boxes. Nucleotide numbering starts from the first nucleotide in the mRNA. Reproduced from Nicot et al., 2005, with permission.

finding of specific cytotoxic T cells and antibodies in infected patients suggests that these proteins are expressed in vivo. Proviral clones mutated in either pX ORF I or II, while fully competent in cell culture, are severely limited in their replicative capacity in a rabbit model of HTLV-1 infection. Emerging evidence indicates that the HTLV-1 accessory proteins are important for establishment of viral infectivity, enhance T lymphocyte activation, and potentially alter gene transcription and mitochondrial function (reviewed in Albrecht and Lairmore, 2002; Kehn et al., 2004; Nicot et al., 2005).

Establishment and Persistence of HTLV Infection in the Host

HTLV-1-infected cells enter the human body via three major routes: mother-to-infant transmission (mainly through breast-feeding), sexual transmission, and parenteral transmission. Recipients of blood transfusions have a high probability of getting infected. In contrast, fresh frozen plasma from seropositive donors does not transmit HTLV-1 (Okochi et al., 1984). Similarly, killing live cells from mother's milk by freezing and thawing abolishes virus transmission (Ando et al., 2004). Virus transmission in vivo thus requires live infected cells. This is in accordance with the results of early in vitro experiments, which found cell-free infection ineffective (Yamamoto et al., 1982; Popovic et al., 1983). HTLV-1 can infect various cell types, including T cells, B cells, bone marrow, cord blood, and synovial cells. Its receptor has been identified as the ubiquitous glucose transporter type 1 (Manel et al., 2005). Efficient transmission necessitates, as noted above, a direct intimate contact between infected and noninfected cells. This is a prerequisite for the formation of the so-called "virological synapse," by which a viral core complex containing the viral RNA is transferred into a new target cell (Igakura et al., 2003). Infectious propagation of HTLV-1 thus is highly efficient. It can be targeted on suitable cells and seems not to depend on released virions, which are highly susceptible to various ways of inactivation by the host's immune system.

Compared to HIV, the replication characteristics of HTLV in vivo differ in several aspects. There is no primary HTLV infection with a high level of virions in plasma. HTLV plasma viremia is also absent at the later stages of infection. RT-PCR for HTLV RNA in plasma is negative even in symptomatic patients. HTLV expression in vivo is also low, and viral transcripts require PCR for detection. Only a small minority of cells, about 1/5,000 PBMC, express mRNA, usually for Tax/Rex (Gessain et al., 1991). In contrast, the proviral load, i.e., the proportion of infected PBMC, can be extremely high, exceeding 30% of the PBMC, or 50% of the $CD4^+$ T cells, in some individuals. The presence of such high levels of HTLV-1-infected cells, apparently in the absence of virions, mRNA, or viral protein in the majority of HTLV-1-infected individuals, initially led to the conclusion that the proviral load was maintained mainly by clonal expansion of infected cells (Wattel et al., 1996). This conclusion was supported by the relative lack of sequence variation both within and between HTLV-1 isolates, which appeared to exclude a major role of the error-prone RT in maintaining the proviral load (Daenke et al., 1990).

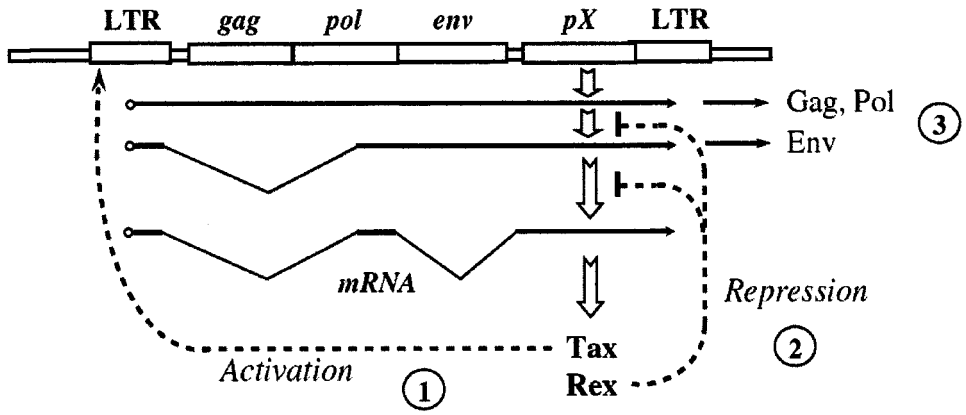


FIGURE 11 Feedback regulation of HTLV-1 gene expression by Tax and Rex. Spontaneous viral expression of Tax from doubly spliced viral transcript. (1) Tax further activates subsequent viral transcription; (2) Rex encoded by the same mRNA as Tax suppresses splicing of viral RNA. (3) Thus, there is accumulation of unspliced mRNAs which express Gag, Pol, and Env proteins on the one side but downregulation of Tax/Rex expression and shutoff of transcription on the other side. Reprinted from Yoshida, 2005, with permission.

The discovery of chronically activated CTL responses, particularly to Tax (Jacobson et al., 1990; Kannagi et al., 1991), and a high titer of anti-HTLV-1 antibodies often including IgM (Nagasato et al., 1991) suggested, however, that in addition to the clonal expansion HTLV expression must be ongoing in chronically infected patients. Therefore, current models propose that during chronic infection there is persistent expression of HTLV, enabling Tax-mediated stimulation of mitosis resulting in clonal expansion. At the same time, virus expression may also lead to new infection of neighboring cells, perhaps preferentially via the immunologically sheltered "virological synapses." Overt expression and release of virions into the bloodstream may, however, be efficiently curtailed by Tax-specific CTL responses, as the expression of Tax occurs prior to that of virion proteins and particle release (Fig. 11). CTL response in turn is subject to host genetic polymorphism, mainly in HLA class 1. In Japan, HLA-A*02 and HLA-Cw*08 were independently and significantly associated with a lower proviral load and a lower risk for HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (reviewed by Bangham and Osame, 2005). Compared to asymptomatic carriers, significantly higher average concentrations of proviral DNA are found in ATLL, HAM/TSP, and other inflammatory HTLV-1-associated disease manifestations (Nagai et al., 1998; Manns et al., 1999b; Bangham, 2003b; Yakova et al., 2005; Silva et al., 2007). Proviral load in asymptomatic carriers correlates strongly with the presence of abnormal lymphocytes resembling malignant ATLL cells (Hisada et al., 1998) and was identified as a significant predictor of subsequent progression to ATLL (Okayama et al., 2004). Interestingly, there is also a strong correlation between *Strongyloides stercoralis* infection and the provirus load, suggesting that strongyloidosis could promote oligoclonal proliferation and development of ATLL (Gabet et al., 2000).

HTLV-Associated Disease and Pathogenesis

HTLV-1-Associated Diseases

Most individuals infected with HTLV-1 remain disease-free carriers throughout their lifetime. In 2 to 6% of infected individuals, however, chronic disease may develop, usually after a long incubation time. Three characteristic disease entities, ATLL, HAM/TSP, and HTLV-associated uveitis (HAU) have been etiologically linked with HTLV-1 infection in adults. Syndromes found associated with HTLV-1 in children predominantly include infective dermatitis and HAM/TSP. Other manifestations less well linked with HTLV-1 infection include various inflammatory diseases like polymyositis, arthritis, infiltrative pneumonitis, Sjögren's syndrome, and in children, persistent lymphadenopathy (Table 3). A general susceptibility to infectious diseases is also frequent.

ATLL

Three decades ago, ATLL was recognized as a new disease in Japan based on characteristic clinical features, origin of the patients from a distinct geographic region in the south

TABLE 3 Diseases associated with HTLV-1 infection[a]

Disease	Association
Adults	
ATLL	+ + + +
HAM/TSP	+ + + +
Uveitis (frequent in Japan)	+ + + +
IDH (rare)	+ + +
Polymyositis, inclusion body myositis	+ +
HTLV-1-associated arthritis	+ +
Pulmonary infiltrative pneumonitis	+ +
Sjögren's syndrome	+
Children	
IDH (frequent in Jamaica)	+ + + +
HAM/TSP (rare)	+ + + +
ATLL (very rare)	+ + + +
Persistent lymphadenopathy	+

[a]Reprinted from Proietti et al., 2005, with permission.

of the country, and a $CD4^+$-T-cell phenotype of the leukemic cells (Takatsuki et al., 1977; Uchiyama et al., 1977). ATLL develops after a long latency in a small fraction (2 to 6% lifetime risk) of HTLV-1-infected individuals that are normally infected shortly after birth (Tajima et al., 1990; Tajima and Cartier, 1995). The mean age at disease onset is in the fifth life decade, and the male-to-female ratio is 1.4:1 (Uchiyama et al., 1977; Tajima et al., 1990; Shimoyama, 1991). Recently, however, acute-type ATLL was diagnosed also in 3 of 8 HTLV-1-seropositive carriers only 6, 9, and 25 months after the start of immunosuppressive therapy given in conjunction with allogeneic liver transplantation (Kawano et al., 2006). ATLL is classified into four clinical types: acute, chronic, smoldering, and lymphoma type. More than 50% are of the acute type, 20% of the lymphoma type, 20% of the chronic type, and about 5% of the smoldering type (Yamaguchi et al., 1983; Kawano et al., 1985; Shimoyama, 1991; Takatsuki et al., 1996; Yamaguchi and Watanabe, 2002).

Acute ATLL characteristically presents with general malaise, fever, cough, dyspnea, abdominal fullness, thirst, and drowsiness. The diagnosis is based on the following criteria: (i) histologically and/or cytologically proven lymphoid malignancy of T-cell type; (ii) abnormal T lymphocytes with deeply convoluted or lobulated nuclei, also referred to as flower cells, present in the peripheral blood (Fig. 12), except in the lymphoma type; (iii) presence of HTLV-1 antibody in serum; and (iv) demonstration of clonality of HTLV-1 proviral DNA in tumor cells, for example, by Southern blotting, which is a definite diagnosis of ATLL (Takatsuki et al., 1977; Shimoyama, 1991; Yamaguchi and Watanabe, 2002). Chronic ATLL is characterized by milder signs and symptoms and a more protracted clinical course. Patients with smoldering ATLL have fewer leukemic cells in their blood and frequently present with skin lesions, such as papules, nodules, and erythema. Lymph node enlargement and splenomegaly in these patients are minimal, and serum lactate dehydrogenase is normal to slightly elevated (Yamaguchi et al., 1983). In lymphoma-type ATLL, the predominant finding is lymph node enlargement.

Major complications of ATLL include hypercalcemia, which eventually develops in 70% of the patients (Prager et al., 1994; Nosaka et al., 2002), and serious opportunistic infections by bacteria, fungi, protozoa, and viruses. As in AIDS, *Pneumocystis jiroveci* pneumonia, *Strongyloides stercoralis*, aspergillosis or candidiasis, and cytomegalovirus pneumonia are common and contribute to the poor prognosis (Shimoyama, 1991; Carvalho and Da Fonseca Porto, 2004; Taylor and Matsuoka, 2005). In 1991, the median survival time reported was 6 months for the acute type, 10 months for the lymphoma type, and 2 years for the chronic type. The corresponding 4-year survival rates were 5% for the acute and lymphoma types, 27% for the chronic type, and 63% for the smoldering type (Shimoyama, 1991). Little progress has been made since then, and the vast increase in knowledge of the molecular biology and oncogenesis of ATLL still awaits translation into clinical benefit (Yamada and Tomonaga, 2003).

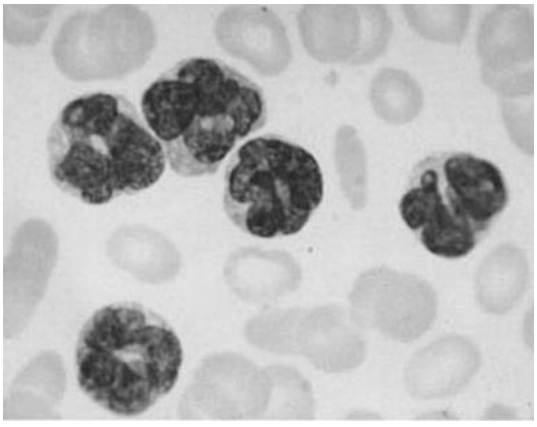


FIGURE 12 Morphology of ATLL cells. Note the typical nuclear indentations (flower cells). Reprinted with permission from Feller and Diebold, 2004.

The pathogenesis of ATLL involves a number of factors and several disease steps (Fig. 13). After initial cell-to-cell transmission, HTLV-1 propagates itself by both de novo infection and Tax-mediated clonal expansion and inhibited apoptosis of infected cells. Because the HTLV provirus integrates at random sites into the cellular DNA, individual infected clones can be detected by Southern blotting, inverse PCR, or once an integration site has been identified, site-specific PCR. During the first 3 to 5 years after seroconversion, a greater number of clones with fewer infected cells is present than is found in long-term carriers (Tanaka et al., 2005). Thus, the clonal proliferations of HTLV-1-infected cells become persistent, and the same clones can be detected at different time points (Etoh et al., 1997; Cavrois et al., 1998). As an example, an HAM/TSP patient developed lymphoma-type ATLL. The ATLL clone was identified in a blood sample obtained before the onset of ATLL, which showed that the same clone was already present during HAM/TSP (Tamiya et al., 1995). Cell clones subsequently converting to malignant ATLL cells were also identified by inverse PCR in patients of a prospective study of HTLV. Such clonal proliferation is directly associated with the onset of ATLL (Okayama et al., 2004). These studies clearly illustrate that HTLV-1-infected clones can progress to a malignant state during the carrier state.

All along the carrier state, infected cells undergo selection by the host's immune system, in particular, cell-mediated immunity (Bangham, 2003a, 2003b), by the genetic and epigenetic environment of proviral integration sites that influences provirus expression, and by other factors. Such selection results in fewer clones, which further expand and predominate in long-term carriers (oligoclonal phase). In leukemic cells, *tax* gene expression is frequently impaired by genetic and epigenetic mechanisms. The resulting loss of Tax expression enables ATLL cells to escape the host's antiviral defenses. On the other hand, ATLL cells must have acquired the ability to proliferate without Tax by intracellular genetic and epigenetic changes (reviewed in Taylor and Matsuoka, 2005).

HTLV-1-associated inflammatory disorders

HTLV-1 is associated with a number of inflammatory disorders in addition to ATLL (Table 3). In contrast to ATLL, which is thought to develop partially due to a lack of secondary deterioration of CTL functions against T cells expressing HTLV-1, the HTLV-1-associated inflammatory disorders are increasingly viewed as resulting from tissue damage triggered by exaggerated HTLV-1-induced T-cell responses (Shimojima et al., 2004).

HAM/TSP. Following identification of HTLV-1 as the etiologic agent of ATLL, a serological association with HTLV-1 was independently reported for patients from the

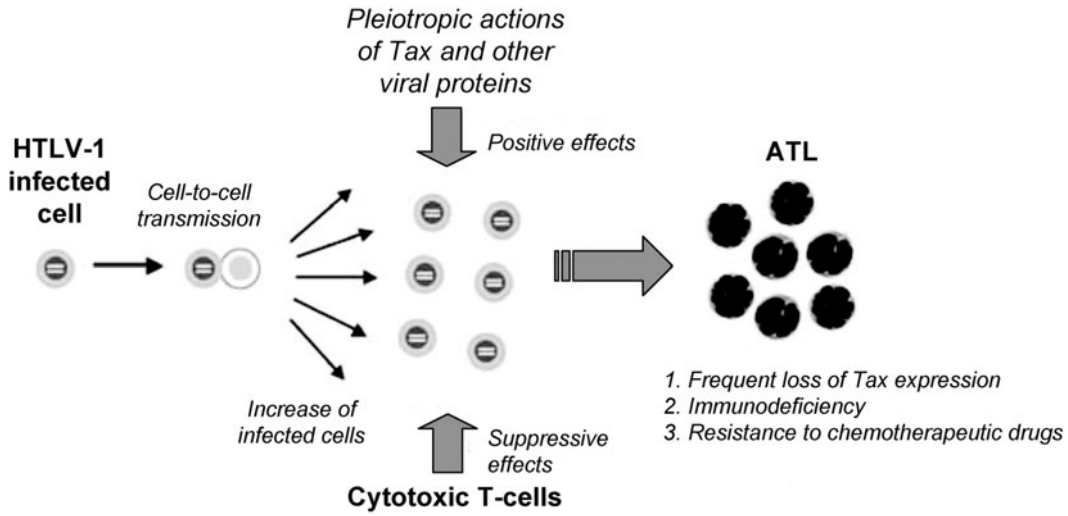


FIGURE 13 Natural course from HTLV-1 infection to onset of ATLL. HTLV-1 is transmitted in a cell-to-cell fashion. After infection, HTLV-1 promotes clonal proliferation of infected cells by pleiotropic actions of Tax and other viral proteins. Proliferation of HTLV-1-infected cells is controlled by cytotoxic T cells in vivo. After a long latent period, ATLL develops in about 5% of asymptomatic carriers. In ATLL, the expression of Tax is inactivated by several mechanisms, suggesting that Tax is no longer necessary in the ATLL stage. Alternatively, alterations and errors in the host genome accumulate progressively during the latent period, finally leading to onset of ATLL. Reprinted from Taylor and Matsuoka, 2005, with permission.

Caribbean who suffered from TSP and for patients from Japan who presented with a myelopathy (Gessain et al., 1985; Osame et al., 1986). Comparative studies subsequently demonstrated that the viruses found in these diseases were genetically indistinguishable from the HTLV-1 strains that cause ATLL (Yoshida et al., 1987). HAM/TSP and ATLL are seen together only rarely in the same patient (Bartholomew et al., 1986; Kawai et al., 1989).

The lifetime risk of developing HAM/TSP among seropositive individuals varies from less than 0.1% in Japan to 1.7 to 7% reported from Africa, the Caribbean, and the United States (reviewed by Taylor, 1998). The mean age at onset is in the fourth decade of life, and the male-to-female ratio is about 1:3. The incubation time extends from years to decades but may occasionally be as short as 18 weeks (Osame et al., 1990). Disease onset is usually slow; patients often have had symptoms for years before the diagnosis is established.

Revised diagnostic criteria based on a WHO definition for HAM/TSP were proposed recently (De Castro-Costa et al., 2006). A definite diagnosis of HAM/TSP thus requires the following. (i) A nonremitting progressive spastic paraparesis with sufficiently impaired gait to be perceived by the patient must be present. Sensory symptoms or signs may or may not be present. When present, they remain subtle and without a clear-cut sensory level. Urinary and anal incontinence may or may not be present. (ii) HTLV-1 antibodies must be present in serum *and* cerebrospinal fluid (CSF), confirmed by WB and/or a positive PCR for HTLV-1 in blood and/or CSF. (iii) A long list of other conditions that can resemble HAM/TSP, comprising, for example, multiple sclerosis, Lyme disease, neurosyphilis, or neurotuberculosis, must be excluded. Criteria for probable and possible HAM/TSP were also proposed.

Pathological examination shows the most prominent changes in the thoracic spinal cord, with atrophy of the cord and thickening of the meninges. The primarily affected white matter shows inflammatory cell infiltrates which, to a lesser degree, also are seen in gray matter (Iwasaki, 1993). These inflammatory infiltrates are initially comprised of both $CD8^+$ and $CD4^+$ T cells, B cells, and foamy macrophages, but later in the disease $CD8^+$ T cells predominate. Through this inflammatory process, myelin and axon loss eventually occurs, and the tissue is replaced by glial proliferation and fibrillary astrocytosis (Iwasaki, 1993; Abe et al., 1999; Jacobson, 2002; Grindstaff and Gruener, 2005). Similar changes are seen in the WKAH rat model after infection with HTLV-1 (Miyatake et al., 2006).

The HTLV-1 proviral load in PBMC of HAM/TSP patients is high; provirus concentrations are 3- to 50-fold higher than in asymptomatic carriers (Kubota et al., 1993; Olindo et al., 2005). HAM/TSP prevalence rose exponentially with the log(proviral load) when the proviral load exceeded 1% of the PBMC (Nagai et al., 1998). A high ratio of proviral loads in CSF cells compared to PBMC, but not the absolute load in either compartment, was found associated with clinically progressive disease and recent onset of HAM/TSP (Takenouchi et al., 2003). Together, these findings suggest that clinical progression of HAM/TSP is associated with increased proliferation in, or increased immigration of HTLV-1-infected lymphocytes into, the CNS. How the presence of HTLV-1 in the CNS leads to HAM/TSP is still unclear, however, and several models are proposed (reviewed by Jacobson, 2002; Bangham, 2003b; Araujo and Silva, 2006). One model centers around a CTL response to Tax-expressing cells within the CNS, leading to coactivation of microglial cells and release of cytokines like tumor necrosis factor alpha that are toxic to the myelin (bystander effects). An autoimmune mechanism induced by molecular mimicry between Tax and a neuron-specific autoantigen, the so-called heterogeneous nuclear RNP A1, and associated with detectable cross-reactive antibodies in HAM/TSP also has been proposed (Lee et al., 2005).

HAU. In 1992, a uveitis of otherwise unexplained etiology in HTLV-1-infected patients was proposed as another disease entity (Mochizuki et al., 1992). HAU patients are of similar age as those with HAM/TSP. They present with a blurred vision of acute or subacute onset, preserved visual acuity in most instances, iritis, vitreous opacities, retinal vasculitis with exudates, and hemorrhages (Takahashi et al., 2000). The condition usually responds well to ocular or oral administration of corticosteroids, although recurrence is observed frequently.

Proviral DNA of HTLV-1 is present in a high percentage of T lymphocytes isolated from the intraocular fluid, suggesting that the intraocular presence of HTLV-1-infected T lymphocytes is due to positive selection rather than coincidence. These T cells are activated and release inflammatory cytokines deemed responsible for HAU, since they can be abrogated by corticosteroid therapy (Sagawa et al., 1995). HAU frequently seems to be associated with a history of Graves' disease (Yamaguchi et al., 1994; Watanabe et al., 1997; Sarui et al., 2002); the combination of the two diseases was associated with a significantly higher proviral load in PBMC. The proviral load also correlated with disease activity in terms of vitreous inflammation and interval to recurrence (Ono et al., 1998).

HTLV-1-associated arthritis. A chronic arthritis in HTLV-1-infected individuals was first described in 1989 (Nishioka et al., 1989). HTLV-1-specific antibodies can be shown in synovial fluids of the affected joints, and proviral DNA was demonstrated in synovial tissues and synovial fluid lymphocytes (Kitajima et al., 1991). Like HAM/TSP and HAU, HTLV-1-associated arthritis is associated with increased proviral DNA levels. Similar to HAU, an increased provirus load was found in the disease-affected tissue (synovial cells) compared to PBMC (Yakova et al., 2005).

Evidence that the arthritis of HTLV-1-infected persons is not due to a mere coincidence with a disease frequent in most countries includes the demonstration that HTLV-1 *env*-pX transgenic mice or rats develop chronic inflammatory lesions with similarity to human rheumatoid arthritis (Iwakura et al., 1995; Abe et al., 2006). The majority of infiltrating T cells in arthritic joints of *env*-pX rats were activated $CD4^+$ T cells, and their transfer into the joints of wild-type rats also induced arthritis.

HTLV-1-associated bronchopneumopathy. Pulmonary complications are more frequent in patients with ATLL than in patients with other hematologic malignancies. They are present in more than 90% of the ATLL patients and include, in addition to leukemic cell infiltration, a variety of opportunistic infections. Patients with inflammatory conditions like HAM/TSP, HAU, or arthritis also have frequent pulmonary complications characterized by T-lymphocytic alveolitis in the absence of leukemic cells or opportunistic pathogens. A similar pulmonary involvement is also detectable in clinically asymptomatic carriers. Characteristically, respiratory symptoms and chest radiographic abnormalities are rare, although 60 to 80% have T-lymphocytic bronchiolitis, alveolitis, or interstitial pneumonia. Chronic sinusitis is also frequent, especially in patients with diffuse panbronchiolitis. Regarding pathogenesis, similar mechanisms as discussed for HAM/TSP are proposed, namely cytotoxic T-cell responses and an inflammatory effect exerted by cytokines released from activated cells (Sugimoto et al., 1998; Seki et al., 2000).

HTLV-1-associated IDH. HTLV-1-associated infective dermatitis (IDH) is a recurrent, infective form of eczema first described in Jamaica in 1966 (Sweet, 1966) and later linked to vertically transmitted HTLV-1 infection (La Grenade et al., 1998). The onset is generally after 18 months of life, and the disease rarely persists until adulthood. IDH is a chronic, relapsing skin infection frequently involving staphylococci or streptococci. It always involves the scalp and may progress to HAM/TSP or ATLL. Most cases of IDH were reported from Jamaica and Brazil. Smaller case series were described in Trinidad and Tobago, Peru, and Senegal. Curiously, in Japan, where the prevalence of HTLV-1 infection is elevated, only two cases of children with IDH have been reported, both of which progressed to ATLL in adulthood (Bittencourt et al., 2006).

Unproven associations of HTLV with other diseases. A large study of the most common hematological diseases in Europe (n = 730 plus 210 controls) reported HTLV-1 infection in 11/67 (17%) patients with a myelodysplastic syndrome (a neoplasia of myeloid cells), 1/26 patients with T-cell non-Hodgkin's lymphoma, and 1/1 patient with T-cell acute lymphocytic leukemia (Karlic et al., 1997), but these findings, in particular regarding the myelodysplastic syndrome, remain unconfirmed (Morselli et al., 1999). Claims that cutaneous T-cell lymphomas are associated with an isolated presence of the HTLV-1 *tax* gene in the absence of antibodies to structural proteins (Manca et al., 1994; Pancake et al., 1995; Pancake et al., 1996) were not confirmed by other groups, even when cutaneous T-cell lymphoma patients from regions of HTLV-1 endemicity or from the same country from which such reports originated were analyzed (Boni et al., 1996b; Bazarbachi et al., 1997; Kikuchi et al., 1997; Wood et al., 1997). Reports of an isolated presence of sequences related to HTLV-1 *tax* continue, however, also in association with autoimmune disorders (Zucker-Franklin et al., 2000; Zucker-Franklin, 2001; Manca et al., 2002; Morozov et al., 2002; Morozov et al., 2005).

HTLV-2-Associated Diseases

HTLV-2 was originally isolated from a T-cell line (Mo-T) derived from a patient with a T-cell variant of hairy cell leukemia (Kalyanaraman et al., 1982). Subsequently, the virus was also isolated from a similar case which upon closer examination demonstrated a coexistence of two different proliferative processes, namely a $CD8^+$-T-cell leukemia with monoclonally integrated HTLV-2 and a B-cell hairy cell leukemia which was negative for integrated HTLV-2 (Rosenblatt et al., 1986).

A pathogenic role of HTLV-2 in malignancies involving $CD8^+$ T cells was supported by the virus' tropism for $CD8^+$ T cells in vitro (Ijichi et al., 1992) and the fact that lymphocytes of HTLV-2-infected patients proliferate in vitro in the absence of antigenic stimulation, similar to cells of HTLV-1-infected individuals (Wiktor et al., 1991). Epidemiological studies have, however, excluded that the typical B-cell form of hairy cell leukemia is associated with HTLV-2 (Hjelle et al., 1991). There is also no further evidence supporting a significant role of HTLV-2 in causing lymphoproliferative diseases. Some evidence links HTLV-2 with HAM/TSP and perhaps with other neurological syndromes (Jacobson et al., 1993; Lehky et al., 1996; Murphy, 1996). It also appears to be associated with an increased incidence of pneumonia and bronchitis, inflammatory conditions such as arthritis, and perhaps, an increased mortality (Murphy, 1996; Roucoux and Murphy, 2004; Jarvis et al., 2005). In general, HTLV-2 pathogenicity is lower than that of HTLV-1 (Feuer and Green, 2005), which may be due to a

lower proviral load (Hisada et al., 2005). The generally lower proviral load may be associated with a comparatively lower efficiency of Tax-2 for viral transactivation, cellular transformation, induction of cell cycle arrest, and suppression of hematopoiesis (reviewed by Feuer and Green, 2005).

Epidemiology and Transmission of the HTLVs

Foci of HTLV-1 infection are found geographically clustered, amounting to about 20 million infected individuals worldwide (Proietti et al., 2005). The geographic distribution of the virus has been defined, with Japan, Africa, the Caribbean islands, and South America as the areas of highest prevalence. Additional regions of endemicity include the Middle East, the Pacific Melanesian islands, and Papua New Guinea (Fig. 14). HTLV-2 is endemic to areas inhabited by Amerindian and Pygmy tribes. More recently, injecting drug users in the United States and Europe have become infected with HTLV-1 and HTLV-2, particularly subtype A, and secondary sexual transmission has introduced the viruses at low levels into the general population and blood donors (Gessain and de The, 1996; Manns et al., 1999a; Roucoux and Murphy, 2004; Taylor et al., 2005).

Like the HIVs, the HTLVs are transmitted by hetero- or homosexual intercourse, from mother-to-child, or by parenteral inoculation. Live HTLV-infected cells are essential in all transmission modes. Mother-to-child transmission occurs with 15 to 30% similar frequency as in untreated HIV-1 infection and occurs predominately in the postnatal period through breast milk. Breast milk transmission seems to be more efficient than for HIV-1 and occurs with a time- or dose-dependent frequency. In one study, overall transmission was 16%. It was 5% among infants breast-fed for up to 3 months and 27% among those breast-fed for over 3 months. Of 78 bottle-fed infants, 13% turned out to be infected, suggesting connatal transmission also (Hirata et al., 1992). In other studies, however, connatal transmission was considerably less frequent (3%). Although HTLV-1-infected cells were detected by PCR in 2.5% of the cord blood samples from HTLV-1-positive pregnancies, this was not associated with infection when the babies were fed formula (Hino et al., 1996). In high-prevalence areas, serologic testing of pregnant women and counseling of those found infected with regard to alternatives to breast-feeding is thus recommended. Transmission by breast milk also depends on its provirus load (Ureta-Vidal et al., 1999; Li et al., 2004).

Transmission by blood products is, in contrast to HIV, strictly cell-associated; the virus is not transmitted by plasma or plasma-derived products (Okochi et al., 1984). Recipients of contaminated blood seroconvert with a 40 to 60% probability and a median seroconversion time estimated at 51 days (Manns et al., 1999a). HTLV screening of blood donors is justified in countries with an elevated prevalence in the general population.

Diagnosis of HTLV Infections

The principles, tools, and problems of HTLV diagnosis are, with some modifications, the same as those for the diagnosis of HIV (see above). Screening is based on tests for HTLV-specific antibodies by ELISA or particle agglutination tests. Confirmatory tests are based on WB or LIA. Since there are many indeterminate WB results, confirmation must be backed by supplemental tests, usually PCR for proviral DNA. Tests for virion components (antigen, viral RNA) in plasma are not to be used, as there is no HTLV plasma viremia.

Screening Tests for HTLV-1 and -2

For HTLV-1 and -2 screening, tests analogous to those reviewed in Fig. 6 for the detection of HIV are used, with

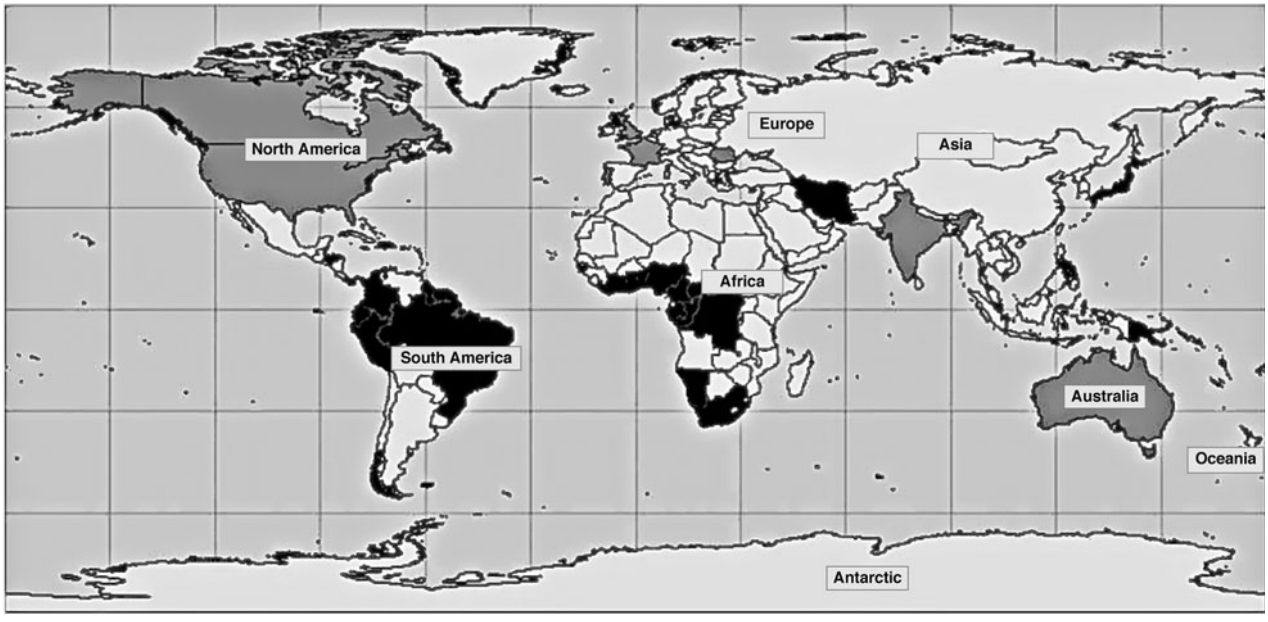


FIGURE 14 Epidemiologic map of HTLV-1. Countries with a prevalence between 1 and 5% in some populations are shown darkly shaded. Countries with a prevalence of less than 1% in some groups, due mainly to immigration from areas of endemicity, are shown lightly shaded. Note that areas of HTLV-1 endemicity do not correspond exactly to the country boundaries shown in the map. For example, HTLV-1 in Brazil, Japan, and Iran is limited to distinct areas within each country. Modified from Proietti et al., 2005, with permission.

the same principal advantages and drawbacks. Since all subtypes of HTLV-1 diverge less than 10% from each other, underdetection of HTLV-1 infection as a result of sequence diversity is not a problem. Similarly, some sequences, like the capsid region of *gag*, are also well conserved between HTLV-1 and HTLV-2, and there is broad serologic cross-reactivity between the two virus types. Due to the low degree of provirus expression and the presumed rapid CTL-mediated elimination of Tax-expressing cells, antibody levels to structural proteins may be low, however. Thus, third-generation tests (DAGS EIA or particle agglutination) are the preferred test formats, as they should provide the highest sensitivity. Tests based on ELISA, particle agglutination, or indirect immune fluorescence are available from various companies (Taylor et al., 1996; Farias de Carvalho et al., 1997; Vrielink et al., 1999; Boni et al., 2004a). For FDA-approved tests refer to http://www.fda.gov/cber/products/testkits.htm. Note that there are considerable differences in the quality of these products, particularly regarding specificity. In low-prevalence populations, a reactive result in an assay of low specificity has a very low positive predictive value (PPV). For example, one recent study involving four different HTLV-1 and -2 EIAs found a specificity above 99% in three of the tests but of only 93% in the fourth test. Even among a high-risk group with a prevalence of 311/100,000, the PPV varied from an unacceptably low 4% (for the test with 93% specificity) to a maximum of 44% for a test with 99.6% specificity. Provided that these assays exhibited the same specificity among blood donors (in whom the prevalence was 0.08/100,000), these PPV would indicate that, with the most specific assay, only 2/10,000 reactive results would indicate a true infection. In contrast, with the least specific assay, only 1/100,000 reactive results would indicate a true infection. These data illustrate the need for both a careful choice of screening assays and of supplementary testing (Petersen et al., 1994; Boni et al., 2004a).

Supplemental Tests for HTLV-1 and -2

WB and LIA

Serologic confirmation of HTLV-1 and -2 infection requires the demonstration of antibodies to both *gag* (p24) and *env* (gp46 and/or gp68 proteins) by WB and/or radioimmunoprecipitation assay (Centers for Disease Control and Prevention and the USPHS Working Group, 1993). In our view, WB is preferable to in-house methods of confirmation like radioimmunoprecipitation assay, since the commercial WB kits are better standardized and much easier to run. WB kits are provided by a number of companies. Of particular interest are strips which contain, in addition to the viral proteins derived from viral lysate, recombinant proteins representing TM of HTLV-1 (which due to a high homology is also detected by antibodies from individuals infected with HTLV-2) and type-specific SU (gp46) of HTLV-1 and HTLV-2. This increases the sensitivity, since the concentration of the gp46 for HTLV-1 and gp68 for HTLV-2 Env proteins on the strips is usually low in kits derived from lysate alone. Based on the pattern of the reactivity, it is frequently, though not always, possible to decide whether infection by HTLV-1 or HTLV-2 is present. With such strips, intense reaction with the *gag* proteins p19 and p24 and the *env* recombinant proteins rgp21 and rgp46 of HTLV-1 satisfies positivity for HTLV-1, and intense reaction with p24, rgp21, and rgp46 of HTLV-2 satisfies positivity for HTLV-2 (Medrano et al., 1997).

Furthermore, the use of LIA strips, which contain standardized concentrations of recombinant proteins and/or synthetic peptides of HTLV-1 and/or HTLV-2 at defined positions and thus can be considered a kind of second-generation WB, presents advantages with respect to both sensitivity and specificity (Zrein et al., 1998; Sabino et al., 1999; Thorstensson et al., 2002).

Still, in many cases WB and LIA do not permit an unequivocal diagnosis, owing to a high percentage of samples with indeterminate results. There is nonspecific reaction not only with lysate-derived natural Gag proteins but also with recombinant Env proteins (Table 4). Some of these indeterminates may have very intense reactions with p19 or p24 and a variety of larger proteins such as p26, p28, p32, p36, p45, and p53. These proteins are present in HTLV-1-infected cells and contain either a p19 or p24 moiety or both (Schupbach and Kalyanaraman, 1989). Reaction with several of these Gag proteins on a WB thus may signify no more than reaction with a single epitope of p19 or p24, respectively (Schupbach et al., 1988). Sometimes, intense *gag* patterns may also be combined with weak reaction to envelope rgp21 and/or $rgp46^{I}$ or $rgp46^{II}$, but even this does not necessarily imply HTLV infection. Weak reactions with recombinant envelope proteins in all possible combinations may also be found in the absence of reaction with Gag proteins. In many instances, confirmation by PCR is thus necessary. This is particularly true for areas or populations in which HTLVs are not endemic. Under such conditions, any suggestive serological result not strongly antibody positive should be confirmed by PCR. Indeterminate reactions are frequent in populations at risk for exposure, such as drug addicts (Medrano et al., 1997), but may also be present in truly infected individuals (Zehender et al., 1996; Caterino-de-Araujo et al., 1998). Infection with the severe acute respiratory syndrome (SARS) coronavirus has recently been identified as a possible cause of false reactivity in both HTLV screening tests and WB (Tsao et al., 2005).

Given the problems with nonspecific bands in WB, alternative diagnostic strategies based on testing with a combination of two sensitive and specific EIAs have been proposed (Thorstensson et al., 2002). Thus, while maintaining a higher overall sensitivity than with the classical EIA-WB combination, an EIA-EIA strategy reduced the frequency of samples with indeterminate results to 2.5%.

PCR

PCR analysis for HTLV-1 and/or HTLV-2 DNA is necessary for all serologically indeterminate results in which antibody reaction to Env proteins (rgp21, $rgp46^{I}$, or $rgp46^{II}$) is present. Antibody reaction with Gag proteins p19 and/or p24 alone, or rgp21 alone, has been found by PCR not to be associated with HTLV infection (Defer et al., 1995). PCR is performed on Ficoll-purified PBMC and frequently uses a sequence of *tax* which is conserved for both HTLV-1 and HTLV-2 and amplified by primers designated SK43/SK44, while the product is detected by probe SK45 (Kwok et al., 1988; Kwok et al., 1990). Differentiation of HTLV-1 and HTLV-2 in samples positive in this initial "screening PCR" is then achieved by amplification of a type-specific region in *pol*. Primers SK110 and SK111 in combination with probe SK112 are used for detection of HTLV-1. The same primers in combination with probe SK188 are employed for HTLV-2. This system also is available as a commercial kit (Amplicor HTLV 1-2 PCR test; Roche Diagnostic Systems) (Vrielink et al., 1997). Alternatively, in-house PCR methods described by various authors can be used for both the screening step and the type differentiation (Vandamme et al., 1997; Salemi et al., 1998; Boni et al.,

TABLE 4 Breakdown of procedures undertaken in a reference lab during repeat testing of HIV-positive samples with additional reactivity or high-negative results in HTLV-1 and -2 ELISA screening[a]

Sample no.	Risk category[b]	ELISA[c]		WB[d]										PBMC PCR HTLV	Culture supernatant			Final diagnosis[f]
				Natural proteins							Recombinant proteins				PERT[e]	RT-PCR		
		Test	OD/CO	p19	p24	p26	p28	p32	p36	p53	rp21E	rgp46E/ HTLV-1	rgp46E/ HTLV-2			HTLV	HIV-1	
1	IDU	PL	25.77	+ + +	+ + +	–	+ +	–	+ + +	+ + +	+ + +	–	+ + +	+				HTLV-2
2	IDU	PL	8.09	+ +	+ + +	–	+	–	+ +	+	+ + +	–	+ + +	+				HTLV-2
3	HET	CR	7.76	+ + +	+ + +	+ + +	+ + +	–	–	–	+ + +	+ + +	–	+				HTLV-1
4	HET	PL	7.37	+ + +	+ + +	–	+ +	–	+ +	+ +	+ + +	–	+ + +	+				HTLV-2
5	IDU	CR	6.16	+ + +	–	–	–	–	–	–	+ + +	–	+ + +	+	+	+,+	–,–	HTLV-2
6	IDU	PL	3.34	+ +	+ + +	–	+	–	+ +	+	+ + +	–	+ + +	+				HTLV-2
7	IDU	AB	2.47	–	–	–	–	–	–	–	–	–	+	–	–,–	–,–	–,–	Neg
8	IDU	AB	1.93	–	+ +	–	–	–	–	–	+ + +	–	+ + +	–	–,–	–,–	–,–	Neg
9	IDU	CR	1.50	–	–	–	–	–	–	–	+ +	–	–	–	–	–	+	Neg
10	HET	AB	1.35	–	–	–	–	–	–	–	–	+	–	–	–,–	–,–	–,–	Neg
11	HET	AB	1.34	–	–	–	–	–	–	–	+	–	–	–	–,–	–,–	–,–	Neg
12	HET	AB	1.30	–	–	–	–	–	–	–	–	–	+	–	–	–	+	Neg
13	IDU	AB	1.26	–	–	–	–	–	–	–	–	+ +	–	–	–	–	–	Neg
14	MSM	AB	1.25	–	–	–	–	–	–	–	–	+	+	–	–,–	–	–	Neg
15	HET	AB	1.13	–	+ +	–	–	–	–	–	–	–	–	–	–	–	–	Neg
16	MSM	AB	1.11	+ + +	–	+ +	–	+ +	–	–	–	–	+					Neg
17	IDU	AB	1.07	–	+	–	–	–	–	–	–	–	–	–	–	–	+	Neg
18	IDU	AB	1.06	–	+	–	–	–	–	–	–	–	–	–	–	–	–	Neg
19	IDU	AB	1.01	–	–	–	–	–	–	–	–	+	–	–	–	–	–	Neg
20	HET	AB	0.87	–	–	–	–	+ + +	–	–	–	–	–	–	–,–	–	–	Neg
21	HET	AB	0.86	+ + +	–	+ +	+ +	–	+ +	–	–	–	–	–	–,–	–	–	Neg
22	IDU	AB	0.83	–	–	–	–	–	–	–	+ +	+	+	–	–	ND	ND	Neg

[a]Reprinted from Boni et al., 2004a, with permission. Symbols: –, no reaction; +, clearly visible, but weak; + +, intermediately strong; + + +, strong. ND, not done.
[b]MSM, homosexual contact; HET, heterosexual contact; IDU, intravenous drug use.
[c]PL, Platelia (Bio-Rad); CR, Cobas (Roche); AB, Abbott HTLV-I/II EIA; OD/CO, optical density/cutoff ratio.
[d]HTLV Blot, version 2.4 (Genelabs Diagnostics, Inc.). Reaction intensities were subjectively rated and are indicated by symbols defined in footnote *a*. Reactions with natural proteins p21E and gp46E were all negative and are not listed.
[e]Symbols summarize results for supernatants sampled twice per week for at least 2 weeks. Symbols separated by a comma represent results of duplicate cultures.
[f]Neg, negative.

2004a). Real-time PCR methods also have been described (Davidson et al., 2006).

Virus Isolation

Compared with PCR, virus isolation is time-consuming and overall more expensive and therefore has little use for mere confirmation of HTLV infection. The procedure is, however, still justified as a research tool for detecting unknown retroviruses that might eventually explain some of the numerous indeterminate serologic results (Boni et al., 2004a). Virus isolation should thus not only be evaluated with HTLV-specific tests (antigen assay or immunofluorescence) but, where available, also by assays for particle-associated RT, preferentially PERT assay (see "RT Assays" in the section on HIV). For virus isolation, Ficoll-purified PBMC are cocultured with phytohemagglutinin-preactivated normal PBMC or cord blood leukocytes in an IL-2-containing medium. Supernatant is analyzed twice weekly for RT. In the case of HTLV infection, the PERT assay usually becomes positive within a few days, while detection of RT by conventional assays may take several weeks. When the PERT assay has become positive, specific tests for HTLV, like p24 antigen assay, PCR, or RT-PCR, can be performed. Differentiation of HTLV-1 and HTLV-2 in virus culture is best achieved by PCR. The use of generic PCR primers and specific probes capable of differentiating between the various PTLVs is helpful in such a situation (Vandamme et al., 1997).

Disease Monitoring by Provirus Quantification

As a high HTLV-1 provirus concentration is associated with the development of various HTLV-associated illnesses and the transmission risk by breast milk (Li et al., 2004), measurement of the HTLV-1 provirus load is often required, although it has not yet been as firmly established for disease monitoring, as is the case with the HIV-1 virion load. Most ATLL cells contain only one provirus copy. As ATLL cells are derived from HTLV-1-infected cells, it is reasonable to conclude that most HTLV-1-infected cells also contain only one provirus. Provirus quantification by quantitative PCR thus can be used for enumeration of HTLV-1-infected cells in vivo. The HTLV-1 provirus load in infected individuals differs more than 1,000-fold among asymptomatic carriers (Etoh et al., 1999).

Treatment of HTLV Infection

During the three decades that have passed since the recognition of ATLL, a variety of treatment approaches has been evaluated (reviewed by Taylor and Matsuoka, 2005). Combination chemotherapy with cyclosphosphamide, adriamycin, vincristine, and prednisolone (CHOP) is still the standard first-line therapy. Many patients experience either partial or complete remission, but its duration is usually short and the median survival time is only 6 months. Intensification of CHOP with etoposide, vindesine, ranimustine, and mitoxantrone was associated with a higher remission rate, but median survival increased only insignificantly. The best outcome of combination chemotherapy, with a median survival time of 13 months, was achieved with an aggressive multidrug approach of high bone marrow toxicity given in combination with granulocyte-monocyte colony-stimulating factor (Yamada et al., 2001). Overall, ATLL survival with various chemotherapy regimens remains poor, with survival in several cohorts of patients presenting predominantly with acute leukemia or lymphoma ranging between 5.5 and 13 months. Supplementation of chemotherapy with alpha interferon and zidovudine as first-line therapy yielded a median survival time of 18 months (Matutes et al., 2001; Kchour et al., 2007). Since ATLL is a monoclonal disease which no longer depends on viral replication, it is unlikely that the effect of zidovudine is based on inhibition of RT; an antineoplastic mechanism is more likely. Also of interest are drugs that appear to induce apoptosis of ATLL cells, like the combination of alpha interferon and arsenic trioxide (Mahieux and Hermine, 2005; Heraud et al., 2006), blockers of NF-κB, and histone deacetylation inhibitors like sodium valproate. Sodium valproate is a drug widely prescribed for epilepsy, bipolar disorders, and migraine and has an excellent safety record. Of interest is that dramatic clearance of both lymphoma and leukemia has been demonstrated in a B-cell malignancy of sheep that is induced by bovine leukemia virus, which is a close relative of HTLV. It is hypothesized that inhibition of DNA deacetylation leads to better provirus expression, thereby resulting in improved immune-mediated elimination of virus-expressing leukemic cells (Achachi et al., 2005). Valproate is also of potential interest for prevention of progression to acute ATLL in patients with the smoldering or chronic forms of ATLL or for carriers still asymptomatic with a high proviral load who are at risk for development of disease. A variety of monoclonal antibodies also has been evaluated.

ATLL cells exhibit a high density of the IL-2 receptor alpha chain (CD25), and treatment with CD25-specific monoclonal antibody (MAb) was capable of inducing remission in a minority of the patients (Waldmann et al., 1988). CD25-specific MAb, meanwhile in humanized form, are also tried in combination with CHOP. Another target of MAb therapy is CD52. A humanized MAb to CD52, Campath-1H, was shown to effectively treat severe combined immunodeficiency mice inoculated with tumor-causing human ATLL cells (Zhang et al., 2003) and showed promise in human patients (Mone et al., 2005).

Finally, both allogeneic and autologous bone marrow transplantation are evaluated as possible treatments of ATLL. A first case of apparent cure was reported in 1996. After a 4-day infusion of cyclophosphamide, etoposide, and doxorubicin, the patient was grafted with bone marrow cells donated by an HTLV-uninfected sister (Borg et al., 1996). Nine years later, in 2005, the patient was still alive and free of disease. In a case series of 10 patients receiving allogeneic hematopoietic stem cell therapy (Allo-SCT) (9/10 from HLA-identical siblings), after receiving total body irradiation and other conditioning agents, the median leukemia-free survival time was >17.5 months. Four patients died, however, and in two others, ATLL relapsed (Utsunomiya et al., 2001). Further experience with this treatment in 40 patients shows that complete remission is achieved in a high proportion of patients. However, there is also a high rate of transplantation-associated complications resulting in an overall median survival of less than 10 months (Fukushima et al., 2005).

Overall, ATLL remains a disease of poor prognosis. Prevention of the disease is thus of paramount importance. Public health intervention with the aim to provide education and counseling of high-risk individuals and populations is required. Avoidance of breast-feeding and the introduction of HTLV screening of all blood donors have led to a significant decline of the carrier rate among the younger generation in Japan (Takatsuki et al., 1996). Given the high kit costs for blood donor screening, a transfer of this strategy to resource-poor settings with HTLV-1 endemicity has so far not been possible. Blood transfusion still represents a risk of HTLV-1 infection for recipients in most African countries as well as for other less-developed areas (Mbanya et al., 2003). Prevention of mother-to-child transmission would likely have a significant impact on the incidence of HTLV-1-associated diseases, but

the benefits of avoiding breast-feeding must be weighed against its risks, namely malnutrition and increased infant mortality. Recommendations to prevent sexually transmitted infections are the same as for the prevention of HIV infection.

APPENDIX 1: SIGNS AND CONDITIONS DEFINING CATEGORY B

Symptomatic conditions in an HIV-infected adolescent or adult that are not included among conditions listed in clinical category C and that meet at least one of the following criteria: (i) attributed to HIV infection or indicative of a defect in cell-mediated immunity; (ii) considered by physicians to have a clinical course or to require management that is complicated by HIV infection.

For example:

- Bacillary angiomatosis
- Candidiasis, oropharyngeal (thrush)
- Candidiasis, vulvovaginal; persistent, frequent, or poorly responsive to therapy
- Cervical dysplasia (moderate or severe) or cervical carcinoma in situ
- Constitutional symptoms, such as fever (38.5°C) or diarrhea for >1 month
- Hairy leukoplakia, oral
- Herpes zoster (shingles), involving at least two distinct episodes or >1 dermatome
- Idiopathic thrombocytopenic purpura
- Listeriosis
- Pelvic inflammatory disease, particularly if complicated by tubo-ovarian abscess
- Peripheral neuropathy

APPENDIX 2: SIGNS AND CONDITIONS DEFINING CATEGORY C (AIDS INDICATOR DISEASES)

- Candidiasis of bronchi, trachea, or lungs
- Candidiasis, esophageal
- Cervical cancer, invasive (added in the 1993 expansion of the AIDS surveillance case definition).
- Coccidioidomycosis, disseminated or extrapulmonary
- Cryptococcosis, extrapulmonary
- Cryptosporidiosis, chronic intestinal (>1-month duration)
- Cytomegalovirus disease (other than liver, spleen, or nodes)
- Cytomegalovirus retinitis (with loss of vision)
- Encephalopathy, HIV related
- Herpes simplex: chronic ulcer(s) (>1-month duration); or bronchitis, pneumonitis, or esophagitis
- Histoplasmosis, disseminated or extrapulmonary
- Isosporiasis, chronic intestinal (>1-month duration)
- Kaposi's sarcoma
- Lymphoma, Burkitt's (or equivalent term)
- Lymphoma, immunoblastic (or equivalent term)
- Lymphoma, primary, of brain
- *Mycobacterium avium* complex or *Mycobacterium kansasii*, disseminated or extrapulmonary
- *Mycobacterium tuberculosis*, any site (pulmonary [added in 1993] or extrapulmonary)
- *Mycobacterium*, other species or unidentified species, disseminated or extrapulmonary
- *Pneumocystis jiroveci* (*carinii*) pneumonia
- Pneumonia, recurrent (added in 1993)
- Progressive multifocal leukoencephalopathy
- *Salmonella* septicemia, recurrent
- Toxoplasmosis of brain
- Wasting syndrome due to HIV

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Chlamydiae

CHARLOTTE A. GAYDOS

33

INTRODUCTION AND TAXONOMY

Chlamydiae are obligate intracellular bacteria which cause many diseases in animals and humans. Originally they were classified as *Chlamydia trachomatis*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, and *Chlamydia pecorum*. *C. psittaci*, formerly a heterogeneous group and primarily pathogens of birds and nonhuman mammals, has been separated into the species, *C. psittaci*, *Chlamydia felis*, and *Chlamydia abortus* (Everett et al., 1999). Previously designated MoPn, the mouse pneumonitis strain is now named *Chlamydia muridarum*, and previously designated GPIC, the guinea pig inclusion conjunctivitis strain is now named *Chlamydia caviae* (Everett et al., 1999). *C. pecorum* is found in ruminants (Essig, 2007). *C. trachomatis* is divided into two biovars, trachoma and lymphogranuloma venereum (LGV) (Mahoney and Chernesky, 2003). The trachoma biovar causes ocular trachoma and urogenital infections. Trachoma is the leading cause of preventable blindness (Mahoney and Chernesky, 2003), while urogenital infections are sexually transmitted. *C. trachomatis* also may be transmitted to infants by infected mothers during birth, causing acute neonatal conjunctivitis and infant pneumonia. *C. trachomatis* infections are the most common reportable sexually transmitted diseases among young adults and adolescents, with an estimated 2 to 3 million new cases per year in the United States (Centers for Disease Control and Prevention, 2003, 2006a; Quinn and Cates, 1992). The LGV biovar (L1, L2, and L3) causes LGV, a more invasive, systemic sexually transmitted disease which is relatively uncommon in the United States (Campbell et al., 2006). Although members of the genus *Chlamydia* cause a number of human diseases, in clinical virology the diagnosis most often requested is that of localized *C. trachomatis* lower genital tract infections (Essig, 2007).

The *C. psittaci* group organisms are very common in domestic mammals and birds. *C. psittaci*, which now only includes the avian strains, affects humans only as a zoonosis, and this diagnosis is usually established serologically (Campbell et al., 2006). *C. pneumoniae* is a common cause of human respiratory disease (Grayston et al., 1986, 1990) and has been linked to atherosclerosis (Grayston, 1993). Serology and culture can be used to diagnose infection (Campbell et al., 2006).

BIOLOGY

During their growth, chlamydiae produce characteristic intracytoplasmic inclusions that can be visualized by Giemsa stains or direct fluorescent antibody (DFA) stains of infected patient material, such as conjunctival scrapings, as well as cervical or urethral epithelial cells. Chlamydiae have a unique developmental cycle, which differentiates them from all other microorganisms (Essig, 2007). They are nonmotile, gram-negative, obligate intracellular bacteria and replicate within the cytoplasm of host cells, forming the characteristic membrane-bound inclusions, which are the basis for some diagnostic tests. They differ from the viruses by possessing both RNA and DNA as well as cell walls that are quite similar in structure to those of gram-negative bacteria. However, they lack peptidoglycan; structural integrity depends on disulfide binding of outer membrane proteins.

Chlamydia in the disease trachoma was first isolated by T'ang et al. in 1957 in chicken embryos (T'ang et al., 1957). The introduction of tissue culture procedures for the isolation of *C. trachomatis* increased the clinical relevance of its detection because the earlier chicken yolk sac procedures used for isolation of the organism were very time-consuming. *C. trachomatis* produces a glycogen-like material while replicating within the cytoplasmic inclusion vacuole, which can be stained with iodine. Cultural diagnosis can be made in tissue culture cells such as McCoy cells in 2 to 7 days after processing of the specimen. Nonculture methods, which evolved in the 1980s, such as DFA techniques and enzyme immunoassays (EIAs), made diagnosis of chlamydial infections widely available (Stamm, 1999). These procedures are being replaced by more sensitive nucleic acid amplification tests (NAATs), which were commercialized during the 1990s and are now the preferred type tests for diagnosis, as recommended by CDC (Gaydos, 2005; Centers for Disease Control and Prevention, 2006b).

Chlamydiae are susceptible to many broad-spectrum antibiotics, possess a number of enzymes, and have a restricted metabolic capacity (Bavoil and Wyrick, 2006). None of these metabolic reactions results in the production of energy. Thus, chlamydiae have been considered energy parasites that use the ATP produced by the host cell for their own metabolic requirements. Many aspects of chlamydial molecular

biology are not well understood, but the sequencing of several of the chlamydia genomes and new proteonomics research have provided researchers with many relevant tools for elucidating the biology of the life cycle (Stephens et al., 1998; Bavoil and Wyrick, 2006).

C. trachomatis strains are sensitive to sulfonamides. *C. psittaci* strains are resistant to the action of sulfonamides and produce inclusions, which do not stain with iodine. *C. pecorum* is quite similar to *C. psittaci*. It can be differentiated by monoclonal antibody or DNA homology testing and is a pathogen of ruminants (Fukushi and Hirai, 1993). *C. pneumoniae* has characteristics similar to *C. psittaci* but shows little DNA relatedness to the other species. The elementary bodies (EBs) of *C. pneumoniae* appear to be pear-shaped rather than round, as are the EBs of other *Chlamydia* species (Grayston et al., 1989).

GROWTH CYCLE

Chlamydiae are phagocytized by susceptible host cells (Bavoil and Wyrick, 2006). Following attachment at specific sites on the surface of the cell, the EB enters the cell through a process similar to receptor-mediated endocytosis and resides in an endosome where the entire growth cycle is completed. The chlamydiae prevent phagosome-lysosome fusion. The inclusion membrane is modified by insertion of chlamydia antigens (Rockey et al., 1995). Once the EB (diameter, 0.25 to 0.35 μm) has entered the cell, it reorganizes into a reticulate body (RB) which is larger (0.5 to 1.0 μm) and contains more RNA. After approximately 8 h, the RB begins dividing by binary fission. Approximately 18 to 24 h after infection, these RBs begin to become EBs by a reorganization or condensation process that is poorly understood. The EBs are then released to initiate another cycle of infection. The EBs are specifically adapted for extracellular survival and are the infectious form, whereas the intracellular metabolically active and replicating form, the RB, does not survive well outside the host cell and is adapted for an intracellular environment. Thus, the defining characteristic of chlamydiae is the unique growth cycle that involves alternation between two highly specialized morphologic forms.

IMMUNOLOGY

The chlamydiae possess group (genus)-specific, species-specific, and type-specific antigens. Most of these are located within the cell wall, but precise structural relationships are not known. The major outer membrane protein contains species-, subspecies-, and serovar-specific antigens (Caldwell and Schachter, 1982). The group antigen, shared by all members of the genus, appears to be a lipopolysaccharide (LPS) with a ketodeoxyoctanoic acid as the reactive moiety (Dhir et al., 1971) and is similar to the LPS of some gram-negative bacteria. The specific antigens of *C. trachomatis* are best recognized by a microimmunofluorescence (MIF) technique (Wang and Grayston, 1984; Wang et al., 1985). The major outer membrane protein molecule has been cloned and sequenced, and species- and serovar-specific antigens have been identified within its variable regions (Stephens et al., 1987; Stephens et al., 1988).

Chlamydiae cause the recruitment of lymphocytes to the site of infection by inducing the release of local host factors, which influence the adhesion cascade of cytokines and adhesion molecules. The regulation of the T lymphocyte response that follows infection is critical to the host response, which may cause the development of chlamydia-associated sequelae, such as infertility (Bavoil and Wyrick, 2006). T-helper type 1 lymphocytes are necessary to eliminate chlamydia, but they and other lymphocytes may also contribute to the pathology of the infection. $CD8^+$ T cells are stimulated following chlamydial infection, and this arm of adaptive immunity is important in the control of the infection, probably through the production of gamma interferon (Bavoil and Wyrick, 2006).

PATHOGENESIS

C. trachomatis is almost exclusively a human pathogen (Grayston and Wang, 1975; Schachter and Dawson, 1978). Serovars within this species cause trachoma (serovars A, B, Ba, and C cause endemic trachoma, the most common form of preventable blindness), oculogenital infections (serovars D, E, F, G, H, I, and K, causing urogenital infections, as well as inclusion conjunctivitis and pneumonia in infants born to infected mothers), and LGV (serovars L1, L2, and L3) (Schachter and Dawson, 1978). Sexual transmission of *C. trachomatis* strains other than LGV has been well studied; serovars D through K have been found to be the major identifiable cause of nongonococcal urethritis in men and can cause epididymitis. Proctitis resulting from rectal infection with chlamydia may occur in either sex. In women, cervicitis is a common result of chlamydial infection, and acute salpingitis and endometritis may occur. Serious sequelae in women can follow untreated chlamydial infections, resulting in pelvic inflammatory disease (PID), ectopic pregnancy, tubal factor infertility, and chronic pelvic pain (Stamm, 1999).

The trachoma biovar is essentially a parasite of squamocolumnar epithelial cells; the LGV biovar is more invasive and involves lymphoid cells. Typical of the genus, *C. trachomatis* strains are capable of causing chronic and clinically unapparent, asymptomatic infections. Because their growth cycle is approximately 48 to 72 h, the incubation period of sexually transmitted infections is relatively long, generally 1 to 3 weeks. *C. trachomatis* causes cell death as a result of its replicative cycle and, thus, is capable of inducing cell damage whenever it persists. However, because there are no toxic effects demonstrated, nor is there sufficient cell death because of replication, the majority of the disease manifestations are due to immunopathologic mechanisms or nonspecific host responses to the organism or its byproducts (Bavoil and Wyrick, 2006). In the absence of adequate therapy, chlamydial infections may persist for several years, although symptoms, if present, usually abate (Molano et al., 2005).

In the previous 5 to 10 years since the genomes of chlamydiae were sequenced, the advent of modern era proteomics, the unraveling of host innate immunity, and new host cell-chlamydia interaction studies, many insights have been gained into how chlamydiae adapt and replicate in their intracellular environment and produce disease. These subjects have been excellently discussed in great detail in the new book, *Chlamydia: Genomics and Pathogenesis* (Bavoil and Wyrick, 2006). Additional detailed discussions include, but are not restricted to, the regulation of gene expression, protein localization, the type III secretion system, the roles of CD4 and CD8 cells in the host response, T lymphocyte trafficking, and new insights into chlamydial pathogenesis (Bavoil and Wyrick, 2006).

Genus-specific proteins can be found in extracts of EBs, and one such protein has been implicated as a potential sensitizing antigen capable of inducing delayed hypersensitivity reactions in the eye and skin of previously infected hosts (Watkins et al., 1986). This protein has been identified as a

heat shock protein (HSP60) which shares antigenic epitopes with similar proteins of other bacteria and is highly conserved in humans (Morrison et al., 1989a; Morrison et al., 1989b; Morrison, 2003). One hypothesis is that repeat infections sensitize the host and local delayed hypersensitivity reactions to HSP60 (at the site of infection) cause host cell damage. Persistent or recurrent infections are associated with the development of fibrosis, scarring, and complications following simple epithelial infections. A common endpoint of these late consequences is scarring of mucous membranes. The genital complications can lead to PID and its late consequences of infertility, ectopic pregnancy, and chronic pelvic pain (Hillis et al., 1997), while ocular infections may lead to blinding trachoma (Schachter and Dawson, 1978). High levels of antibody to HSP60 have been associated with tubal factor infertility, ectopic pregnancy (Wagar et al., 1990; Toye et al., 1993), and scarring trachoma (Peeling et al., 1998).

C. pneumoniae infects the respiratory tract (Grayston et al., 1993). Pneumonia and bronchitis are the most frequently recognized illnesses resulting from infection. Approximately 10% of cases of pneumonia and approximately 5% of bronchitis and sinusitis cases in adults have been attributed to *C. pneumoniae* (Grayston, 1992). Asymptomatic infection or slightly symptomatic illnesses are the most common outcome of infection. *C. pneumoniae* also has been associated with other acute and chronic diseases such as purulent sinusitis, otitis media with effusion, endocarditis, bronchitis, asthma exacerbation, chronic obstructive pulmonary disease, erythema nodosum, Guillain-Barré syndrome, reactive arthritis, Reiter's syndrome, and coronary heart disease (Kuo et al., 1995; Gaydos and Quinn, 1999).

EPIDEMIOLOGY

C. trachomatis Infections

Trachoma

The World Health Organization estimates that approximately 6 million people have been blinded by trachoma and that hundreds of millions live in areas of endemicity (Thylefors et al., 1995). The areas of the world where trachoma is hyperendemic are North Africa and sub-Saharan Africa, the Middle East, drier regions of the Indian subcontinent, and Southeast Asia. Foci of trachoma persist in Australia, the South Pacific, and Latin America.

In areas of hyperendemicity, the prevalence of trachoma is essentially 100% by the second or third year of life. Active disease is most common in young children, who are the reservoir of the disease. By adult life, active infection is infrequent, but the sequelae of the disease result in blindness. In such areas, trachoma constitutes the major cause of blindness. Worldwide, trachoma is considered the most common cause of preventable blindness.

Genital Infection

C. trachomatis is the most common sexually transmitted bacterial pathogen. Estimates of annual incidence are approximately 90 million infections worldwide, with 3 to 4 million occurring in the United States (Weinstock et al., 1994; World Health Organization, 2001; Centers for Disease Control and Prevention, 2006a). *C. trachomatis*, like other genital pathogens, has been found with increased frequency among individuals who are younger, nonwhite, unmarried, and of lower socioeconomic status. In the United States, most of the estimated 2 million cases of acute urethritis are nongonococcal, and *C. trachomatis* is implicated as causing 30 to 50% of these cases (Hook and Handsfield, 1990).

Epididymitis, a severe complication of urethritis, occurs in approximately 500,000 men per year in the United States, and approximately half of these cases are caused by *C. trachomatis*. *C. trachomatis* can be identified in 20 to 30% of men with gonorrhea. The syndrome "postgonococcal urethritis" is mainly caused by the chlamydial organism following treatment for the gonococcal infection, since the concomitant infection with *C. trachomatis* does not respond to the treatment for gonorrhea. Men with gonorrhea are now cotreated for chlamydia unless chlamydia is ruled out by specific testing (Lyss et al., 2003). Chlamydial and gonococcal infections may also occur together in women (35 to 45% dual infection is not uncommon), and inadequate treatment to cover chlamydia may be followed by salpingitis. This condition, usually called PID, is the most important consequence of genital chlamydial infection. Late consequences of PID include tubal factor infertility and ectopic pregnancy; chlamydial PID may cause more than 50% of these conditions. A silent salpingitis without overt symptoms may occur and still produce sufficient tubal damage to cause the late consequences.

C. trachomatis infections may be persistent and asymptomatic ("latent" or "silent" infection) for prolonged periods of time. For example, infants have been documented to shed organisms from conjunctivae for more than 2 years postdelivery. Similarly, women with untreated cervical infection have been culture or PCR positive for over 15 months (Molano et al., 2005). Reinfection with chlamydia also has been associated with more adverse outcomes, and some investigators have hypothesized that susceptibility to reinfection, as a result of better, early diagnosis and treatment resulting from chlamydia control programs, may interfere with the effects of natural immunity on population-based susceptibility to infection (Brunham et al., 2005).

Approximately 20 to 30% of infants exposed to *C. trachomatis* in the birth canal will develop conjunctivitis and 10 to 15% will develop pneumonia (Centers for Disease Control and Prevention, 2006b). Conjunctivitis usually develops at 5 to 19 days of life. Infant pneumonia may develop from 2 weeks to 4 months of age. *C. trachomatis* has been estimated to cause 20 to 30% of pneumonia cases in infants less than 6 months of age.

C. psittaci Infection (Psittacosis)

Psittacosis is a relatively rare disease in the United States. In 1994, 38 cases were reported (0.2/1,000,000 population) (Centers for Disease Control and Prevention, 1995). Since 1996, fewer than 50 confirmed cases have been reported in the United States each year (http://www.cdc.gov/ncidod/dbmd/diseaseinfo/psittacosis_t.htm). Many more cases probably occur than are reported. Severe pneumonia requiring intensive care support, endocarditis, hepatitis, and neurologic complications may occasionally occur (Schaffer, 1985). Fatal cases have been reported. Infection is acquired by inhaling dried secretions from or through direct contact with infected birds. The incubation period is 5 to 19 days but can range from 4 to 28 days. Although all birds are susceptible, pet birds (parrots, parakeets, macaws, and cockatiels) and poultry (turkeys and ducks) are the most frequently involved in transmission to humans, although virtually all avian species are potential reservoirs. The risk of exposure is greatly increased in occupations such as poultry breeding and processing, where handling birds is common, as well as

in households with pet birds. Transmission of infection occurs by the airborne route either by direct contact with birds or by inhalation of dust contaminated with excreta of infected birds. Transmission from person to person has been suggested but not proven. Transmission to humans with other chlamydia species affecting nonhuman mammals is rare, with only a few cases of abortion reported in pregnant women who worked with *C. abortus*-infected sheep.

C. pneumoniae Infection

Studies in many countries found 20 to 80% of adults with *C. pneumoniae* antibody, and in most countries, the prevalence is 50% or more (Grayston et al., 1990). Antibody is uncommon under the age of 5 years, and then age-specific prevalence increases rapidly from 5 to 20 years. The prevalence then increases slowly but steadily throughout life. In Seattle, men over 60 have a prevalence of 70%. Rates are higher in men than in women after the age of 20. There is some loss of antibody noted 3 to 5 years after the first infection, so the high rates of antibody after age 70 suggest that virtually everyone is infected at some time in their lives. Reinfections can occur. There is no evidence for other human-to-human transmission of *C. pneumoniae* infection. Epidemics have been studied in military recruits in Finland (Saikku, 1992) and in one adjacent civilian community (Kleemola et al., 1988). Attack rates varied from 60 to 80 per 1,000 men. Transmission was slow, with epidemics lasting as long as 6 months.

Many studies have associated *C. pneumoniae* with coronary heart disease starting in 1992 with a serological association (Saikku et al., 1992). These studies progressed over more than a decade with studies covering associations by serology (Saikku et al., 1988), electron microscopy (Shor et al., 1992), PCR (Boman et al., 1998), and culture of the organism from atheromas (Jackson et al., 1997). Secondary prevention trials using antibiotic treatment of individuals who had previous heart disease have not been successful (Dunne, 1999; Grayston et al., 1999).

LABORATORY DIAGNOSIS

Collection of Specimens

For cytological studies, impression smears of involved tissues or scrapings of involved epithelial cell sites should be appropriately fixed (methanol may be used for DFA stains and Giemsa stains). It is imperative that samples be collected from the involved epithelial cell sites by vigorous swabbing or scraping. This is also true for culture isolation attempts. Purulent discharges are inadequate and should be cleaned from the site prior to sampling.

For *C. trachomatis* infections of humans, which are diagnosed by culture, DFA, or EIA methods, the involved mucous membranes should be vigorously swabbed or sampled by scraping. Thus, the conjunctiva would be sampled for trachoma-inclusion conjunctivitis, the anterior urethra (several centimeters into the male urethra) would be sampled for urethritis, and the cervix (within the endocervical canal) would be sampled for cervicitis. As these strains appear to infect only columnar or squamocolumnar cells, cervical specimens must be collected at the transitional zone or within the os. The organism also can infect the urethra of the female, and it may improve recovery rates if another sample is collected from the urethra and sent to the laboratory for testing in the same tube with the cervical sample. For women with salpingitis, the samples may be collected by needle aspiration of the involved fallopian tube or endometrial specimens may yield the agent. The rectal mucosa, nasopharynx, and throat may also be sampled. For infants with pneumonia, swabs may be collected from the posterior nasopharynx or the throat.

Culture Isolation

The recommended procedures for primary isolation of chlamydiae use cultured cells for the growth of the organism. The most common technique involves inoculation of clinical specimens by centrifugation into cycloheximide-treated McCoy cells (Campbell et al., 2006). Human cells such as HEp2 cells may be more sensitive for isolation of both *C. trachomatis* and *C. pneumoniae*. HL or HEp2 cells are the preferred cell lines of choice for isolation of *C. pneumoniae* (Cles and Stamm, 1990; Hyman et al., 1995). *C. psittaci* will grow in cell culture, in yolk sac, and in mice by a variety of routes of inoculation. *C. psittaci* organisms present a threat to technicians, as laboratory infections have occurred, so caution should be used when attempting isolation.

Culture is mostly performed now only by research or State Health Laboratories, having been mostly replaced by NAATs; however, it is still the recommended method for use in cases of sexual abuse and medico-legal cases (Centers for Disease Control and Prevention, 2006b). For routine (non-medico-legal) use, the CDC now recommends NAATs as the test of choice for the detection of *C. trachomatis* because of their increased sensitivity. In future years, the NAATs may be recommended for medico-legal cases as well; however, because of their less than perfect specificity, at least two assays will probably be required to confirm a positive result. This approach will be useful for treatment purposes, but it remains to be determined whether the legal recommendations will change in favor of NAATs instead of culture (Black et al., 2006). Currently, culture is also FDA cleared and recommended by CDC for testing rectal and pharyngeal samples for chlamydia. However, the use of NAATs which are verified as accurate in a research study by individual laboratories is gaining wide acceptance in public health and will probably become the assay of choice, once FDA clearance is achieved for commercial NAATs (Kent et al., 2005).

Nonculture Methods

Nonamplified Methods

There are a number of ways to detect chlamydiae directly in clinical specimens. These include (i) staining of patient clinical specimens for chlamydia EB using monoclonal antibodies (DFA) or stains such as Giemsa (Taylor et al., 1984; Uyeda et al., 1984; Lidner et al., 1986), (ii) direct antigen detection in EIAs (Gaydos et al., 1990; Clark et al., 1992; Chan et al., 1994; Sanders et al., 1994), and (iii) nucleic acid probe hybridization (Clarke et al., 1993; Warren et al., 1993; Stary et al., 1994). Some of the procedures have been in use for many years. For example, direct microscopic examination for organisms in impression smears of infected avian tissues for *C. psittaci* is still a useful procedure.

Fluorescent Antibody Staining

Fluorescein-conjugated monoclonal antibodies are commercially available and are routinely used in some laboratories to identify chlamydial inclusions in infected cell cultures (Stamm, 1999). These antibodies may be used directly on clinical specimens as well. Species-specific antibodies to *C. trachomatis* are recommended for staining of clinical specimens suspected of containing those organisms. Genus-specific antibodies may

be used for infected cell cultures, but they are less suited for use in direct detection in clinical specimens because of the irregular staining of the LPS in the EB. *C. pneumoniae*-specific monoclonal antibodies appear to be better able to detect that organism in infected cell cultures, as the infectivity of the organism appears to be relatively low. The DFA stain is somewhat less sensitive than isolation in culture but is faster and less expensive and may represent an alternate method of diagnosing chlamydial infections in settings where cell culture isolation is not available, and NAATs are too expensive. The specimen swab is rolled onto a glass slide, air dried, and fixed with methanol. Fluorescein-conjugated monoclonal antibody is applied to the slide. After incubation, a coverslip is placed with mounting medium and read with an epifluorescent microscope under ×1,000 magnification for the presence of EBs. DFA requires a trained microscopist and has a sensitivity of 80 to 85% with a specificity of 98 to 99% compared to culture (Mahony and Chernesky, 2003).

EIA

Most EIAs use polyclonal or monoclonal antibodies to detect chlamydial LPS in clinical specimens. Antigen detection using EIA was widely performed before the advent of molecular tests (Black, 1997) and is still the most prevalent nonculture detection test for *C. trachomatis* (Black, 1997). There are several commercially available EIAs, and these tests can detect all species of chlamydia but have been most extensively validated for urogenital *C. trachomatis*. Compared to newer molecular assays, the sensitivity of the EIAs ranges from approximately 53 to 76%, with specificities of about 95%. Because older nonculture tests, such as DFA and EIA, were compared to culture as a gold standard, the sensitivities reported in the older literature are probably overestimates of true sensitivity. A meta-analysis, which adjusted the sensitivities of such assays based on a sensitivity of culture of 85% has been reported (Howell et al., 1998). Table 1 shows a comparison of the sensitivities and specificities of diagnostic assays available for the detection of *C. trachomatis* in clinical specimens. Methods for improving the sensitivity of EIA include retesting of samples close to the cutoff value (50% below the positive value or "negative gray-zone value") and retesting these negative gray-zone samples by DFA or amplified testing (Beebe et al., 1993). These tests are also generally less sensitive than cell culture performed under ideal settings, but as with the DFA procedures, there are advantages: specimens do not have to be maintained on ice and can be collected from clinic settings distant from the processing laboratory. The EIA is easy to perform and suitable for testing many specimens for high-volume laboratories. Except for the lower cost per test, there is no reason for routine use of EIAs, since NAATs offer far superior performance.

The first commercial, molecular test for *C. trachomatis* was the nucleic acid probe hybridization test (GenProbe, San Diego, CA), which has been widely used, replacing the EIA in many large-volume laboratories (Clarke et al., 1993; Black et al., 2002). It uses DNA-RNA hybridization to detect chlamydial RNA in clinical samples. The sensitivity ranges from 65 to 83%, which is equal to or higher than that of the more-sensitive EIAs, and it is specific (99%) (Black et al., 2002).

The Digene hybrid capture II CT-ID test, (Digene, Silver Spring, MD) can also be used for the detection of *C. trachomatis*. This test does not amplify the nucleic acid but amplifies the detection signal. It has had limited evaluation, but in one study with cervical specimens, the sensitivity and specificity were 95.4% and 99.0%, respectively (Girdner et al., 1999). It is not approved for use with urine or vaginal samples.

As described in "Culture Isolation" above, none of these nonculture assays are recommended where diagnosis may be required for medico-legal purposes (such as instances of sexual abuse or rape) because of concerns about the limitations of less than 100% specificity or for use with rectal or pharyngeal samples. Additionally, none of these nonamplified, nonculture methods are sensitive enough to be used for urine or vaginal swab specimens.

NAATs

NAATs are used most frequently by laboratories at the present time and are the recommended test of choice by the

TABLE 1 Sensitivity and specificity of diagnostic tests for the detection of *C. trachomatis*

Diagnostic method (commercial test) and sample type	Sensitivity (%)	Specificity (%)
Tissue culture	70–85	100
DFA	80–85	>99
EIA	53–76	95
Hybridization (Pace2)	65–83	99
PCR (Cobas)		
Cervical swab	89.7	99.4
Female urine	89.2	99.0
Male urine	90.3	98.4
SDA		
Cervical swab	92.8	98.1
Female urine	80.5	98.4
Male urine	93.1	93.8
TMA		
Cervical swab	94.2	97.6
Female urine	94.7	98.9
Male urine	97.0	99.1
Male urethral swab	95.2	98.2

CDC for the diagnosis of C. *trachomatis* infection (Centers for Disease Control and Prevention, 2006b).

Individual NAATs for Chlamydia

In the early 1990s, the usefulness of PCR was recognized for its ability to detect difficult-to-grow pathogens, and research studies were published for the detection of chlamydia (Bobo et al., 1990; Holland et al., 1990). Soon, commercial assays were developed and FDA cleared for use, and C. *trachomatis* was the very first organism for which there was a commercially available PCR assay (Jaschek et al., 1993). Now there are many published studies using several different types of NAATs; new technologies are commercially available that can detect chlamydia as well as *Neisseria gonorrhoeae* (Bauwens et al., 1993; Quinn et al., 1996; Martin et al., 2000; Van der Pol et al., 2000; Van der Pol et al., 2001; Gaydos et al., 2003). Because of their greatly expanded sensitivity, NAATs were the first tests that were able to be used with urine samples and include PCR (Amplicor; Roche Molecular Diagnostics, Indianapolis, IN), transcription-mediated amplification (TMA) (Aptima Combo2; GenProbe, San Diego, CA), and strand displacement amplification (SDA) (ProbTec; Becton Dickinson, Sparks, MD). These methods offer expanded sensitivities, usually well above 90%, while maintaining very high specificity (Boyadzhyan et al., 2004; Gaydos et al., 2004).

PCR

The sensitivity of PCR for chlamydia in clinical trials was 89.7% for endocervical samples, 89.2% for female urine specimens, 88.6% for male urethral swabs, and 90.3% for male urine specimens, as shown in Table 1 (Van der Pol et al., 2000). This test is available as a microwell format as well as an automated method (Cobas).

SDA

SDA sensitivities for chlamydia of 92.8% for cervical swabs, 80.5% for female urine, 94.6% for male urethral swabs, and 94.5% for male urine were reported in the clinical trial for FDA clearance, as shown in Table 1 (Van der Pol et al., 2001).

TMA

The Aptima Combo2 assay, with hybrid capture technology, showed increased sensitivity ranging from 94.2 to 97.0%, as shown in Table 1 (Gaydos et al., 2003). The somewhat lower specificities determined in the clinical trial may be artificially low, since this assay appears to be slightly more sensitive than other NAATs and confirmation of uniquely positive samples by another NAAT can be problematic. Unique positives not confirmed by a test of lower sensitivity are often confirmed as true positives by another primer set targeting a different gene using this assay (Gaydos et al., 2003; Gaydos et al., 2004; Boyadzhyan et al., 2004). This assay and the "stand alone" chlamydia test (Aptima CT) are the only NAATs FDA cleared for use with self-collected vaginal samples, but only when the samples are collected in a clinic setting (Schachter et al., 2003; Schachter et al., 2005a). Public health officials are hopeful that self-obtained collection of vaginal swabs at home will be FDA cleared in the near future, which would make outreach sampling for chlamydia beyond the clinic possible. In research settings, such home collection of self-obtained vaginal swabs appeared to be acceptable, feasible, and accurate when NAAT was used (Gaydos et al., 2006a; Gaydos et al., 2006b; Hobbs et al., 2008).

Because of potentially lower positive predictive values (i.e., <90%) for NAAT results in low-prevalence populations, where the test specificity is less than 100% (Zenilman et al., 2003), the CDC has recommended that a confirmatory test should be considered for a person with a positive screening test (Centers for Disease Control and Prevention, 2002). CDC suggested that approaches might include testing a second specimen with a different test using a different target, testing the original specimen with a different test that uses a different target or format, repeating the original test on the original specimen with a blocking antibody or competitive probe, or repeating the original test on the original specimen (Centers for Disease Control and Prevention, 2002). The necessity of confirmatory testing for NAATs has been controversial; some studies have suggested that confirmation should not be performed (Schachter et al., 2005b; Schachter et al., 2006).

Other Types of Tests for Chlamydia (Point-of-Care Tests)

Several rapid point-of-care tests have been introduced. These are designed to be performed while the patients wait for results and include individual single-use tests, such as the optical immunoassay (OIA; Biostar, Inc., Boulder, CO) (Pate et al., 1998). Several older point-of-care tests based on EIA-type assays exist, which have a lateral flow platform or cartridge, but these tests have low sensitivities (i.e., 50 to 70% or less) compared to other, newer laboratory-based tests and cannot be recommended for general use. Efforts are under way to improve the sensitivity of these rapid tests, which could make them practical in outreach venues, such as homeless shelters and detention sites.

Serology

The most widely used serological test for diagnosing chlamydial infections is the genus-specific complement fixation (CF) test. It is useful in diagnosing psittacosis, in which paired sera often show fourfold or greater increases in titer. About one-third of patients with other evidence of C. *pneumoniae* infection will also show rising CF titers. Single-point titers greater than 64 may support a diagnosis of LGV. With LGV it is difficult to demonstrate rising antibody titers, since the nature of the disease results in the patient being seen by the physician after the acute stage. Any titer above 16 is considered significant evidence of exposure to chlamydiae. There are some commercially available serologic tests based on measurement of antibodies reactant to chlamydial inclusions in cell culture or EIA using chlamydial antigens. These tests are often described as "specific" by the manufacturers. However, the inclusions contain LPS, and genus-specific cross reaction will occur. None of these tests are recommended for routine use. None has a high predictive value in diagnosis of uncomplicated lower genital infection. The CF test is not useful in diagnosing trachoma-inclusion conjunctivitis or the related genital tract infections, and it plays no role in diagnosing neonatal chlamydial infections.

The MIF test method is a much more sensitive procedure for measuring antichlamydial antibodies (Wang et al., 1979). It may be used in diagnosing psittacosis and C. *pneumoniae*, in which paired sera show rising immunoglobulin G (IgG) titers (and often IgM antibody). With LGV it is again difficult to demonstrate rising titers, but single-point titers in active cases usually have relatively high levels of IgM (>32) and IgG (2,000) antibodies. Trachoma, inclusion conjunctivitis, and the genital tract infections may be diagnosed by the MIF technique if appropriately timed paired

acute- and convalescent-phase sera are obtained. However, it is often difficult to demonstrate rising antibody titers, particularly in sexually active populations, since many of these individuals are seen for chronic or repeat infections.

Serology is particularly useful in diagnosing chlamydial pneumonia in neonates. In this case, high levels of IgM antibody are regularly found in association with disease (Schachter et al., 1982; Schachter et al., 1986). IgG antibodies are less useful because the infants are being seen at a time when they have considerable levels of circulating maternal IgG, since all of these infections are acquired from the infected mother, who is seropositive. It takes between 6 and 9 months for maternal antichlamydial antibodies to disappear from the infant. Infants older than that age may be tested for determination of prevalence of chlamydial infection without fear of the confounding effects of maternal antibody. Infants with inclusion conjunctivitis or respiratory tract carriage of chlamydiae without pneumonia usually have very low levels of IgM antibodies. Thus, a single titer of 32 or greater may support the diagnosis of chlamydial pneumonia.

With *C. pneumoniae* infection, seroconversion may take more than 4 weeks, thus requiring a delay in collection of convalescent-phase sera. However, a consensus statement from researchers in the field declared that seroconversion was the most reliable indicator of a recent *C. pneumoniae* infection (Dowell et al., 2001).

PREVENTION AND TREATMENT

Although there has been considerable effort made to develop a chlamydial vaccine, at this writing there is no effective vaccine for any human chlamydial disease. Prevention is based on treatment of those infected to prevent transmission and complications.

C. psittaci Infection (Psittacosis)

Control of psittacosis depends on control of avian sources of infection. The 1929 pandemic of psittacosis was stopped by banning shipment or importation of psittacine birds. An effective method for controlling psittacosis in parakeets or other seed-eating birds has been developed—a chlortetracycline-impregnated seed. Other birds receive prophylactic treatment via a tetracycline-containing mash. Current requirements for quarantine of imported birds call for 30 days of treatment.

For treating human cases, the antibiotic of choice is tetracycline in a dose (for adults) of at least 250 mg 4 times a day and continued for at least 3 weeks to avoid relapse. Severely ill patients may need measures for cardiovascular and respiratory support. Erythromycin (500 mg 4 times a day orally) is an alternative therapy.

C. trachomatis Infections

Trachoma

There is growing evidence that community-wide treatment with oral azithromycin may help control blinding trachoma (Schachter et al., 1999). Standard treatment of cases in areas of endemicity has been based on long-term (6 weeks), daily topical application of 1% tetracycline ophthalmic ointment.

Genital Infection

Control of genital infections will be based on screening and treatment programs. In addition to reducing the prevalence of infection, these programs prevent expensive complications and have been shown to be cost-effective. Thus screening and treatment for pregnant women will prevent postpartum endometritis and pneumonia in infants (Schachter et al., 1986). Screening and treatment in a family planning clinic will prevent PID (Scholes et al., 1996).

The treatment of choice is a single 1-g oral dose of azithromycin, which has been shown to be as effective as the 7-day doxycycline regimen (Martin et al., 1992). Tetracyclines have been the mainstay of therapy (Centers for Disease Control and Prevention, 2006b). Tetracycline hydrochloride or oxytetracycline 500 mg 4 times daily, minocycline 100 mg twice daily, and doxycycline 100 mg twice daily are equally effective oral treatments for uncomplicated infection in men and women.

C. pneumoniae Infections

Grayston and colleagues found that both tetracycline and erythromycin are effective against *C. pneumoniae* in vitro; they recommended either drug in a dosage of 2 g daily for 10 to 14 days or 1 to 1.5 g daily for 21 days, but stated that relapses may occur even after this intensive treatment (Grayston et al., 1993).

INSIGHTS AND THE FUTURE

The next few years could provide important movement in controlling chlamydial infections and their complications. The potential for control of blinding trachoma is exciting. The importance of the findings that first-catch urine specimens from males and females and vaginal swabs are suitable specimens for diagnosis of genital infection using NAATs cannot be overstated. Exquisitely sensitive and specific noninvasive diagnostic tests will provide the basis for public health screening programs. Together with single-dose treatment, there is a chance for major success.

There are still major needs, which include a protective vaccine, rapid tests to diagnose genital infection to speed up treatment of infected individuals, better diagnostics for *C. pneumoniae*, more information on the real clinical spectrum of *C. pneumoniae* infection, and more information on chlamydial biology, especially on protective immunity.

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Rodent-Borne Viruses

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Rodents are hosts of several pathogens that afflict humans (zoonotic diseases). Rodent-borne agents range from bacterial and rickettsial microbes, such as the plague bacillus (*Yersinia pestis*), *Borrelia* spp., and *Ehrlichia* spp., to parasites (*Babesia microti*). Small mammals, including rodents, may participate in the sylvatic cycles of some arboviruses, but rodents serve as the primary reservoirs of two major groups of medically important viruses: those of the family *Arenaviridae* and those of the genus *Hantavirus*, family *Bunyaviridae*. For both groups of viruses, there is evidence for coevolution with their rodent hosts. Unlike many zoonotic pathogens, hantaviruses and arenaviruses do not require or use an arthropod vector to maintain infection in reservoir populations or to infect humans.

Viruses of the families *Bunyaviridae* and *Arenaviridae* exhibit no cross-reactivity at the antigenic level and no significant nucleotide homology, but the similarities among members of the genus *Hantavirus* and those of the family *Arenaviridae* are striking nonetheless. Both have segmented, negative-sense or ambisense single-stranded RNA genomes (Buchmeier et al., 2007; Schmaljohn and Hooper, 2001; Schmaljohn and Nichol, 2007; Zheng et al., 2007). Both are found in rodent populations, often in very focal distribution, and confine themselves predominantly to one or two closely related species within the families *Muridae* and *Cricetidae*. Taxonomic issues still remain in attempts to validate the status of several of the carrier host species (Salazar-Bravo et al., 2002a). The same rodent host may harbor both a hantavirus and an arenavirus, as is the case with the hispid cotton rat *Sigmodon hispidus*, which serves as host to the presumably apathogenic Tamiami arenavirus, the Muleshoe hantavirus, and Black Creek Canal hantavirus (BCCV), an etiologic agent of hantavirus cardiopulmonary syndrome (HCPS) (Calisher et al., 1970; Rollin et al., 1995; Rawlings et al., 1996). The short-tailed cane rat *Zygodontomys brevicauda* is a host to Calabazo hantavirus as well as to the highly pathogenic Guanarito arenavirus (GTOV), the cause of Venezuelan hemorrhagic fever (Fulhorst et al., 1999; Vincent et al., 2000). The list of recognized species has been increasing steadily for both virus groups (Schmaljohn and Hjelle, 1997; Enria and Pinheiro, 2000; Salvato et al., 2006). For both, closely related virus species can exist sympatrically in association with specific reservoir species without influencing one another's evolution (Rawlings et al., 1996). Transmission to humans, and possibly also among the reservoir rodents, is believed to occur through contaminated aerosols of rodent urine, feces, or saliva (Tsai, 1987; Padula et al., 2004; Fulhorst et al., 2007). There are similarities in the diseases caused by arenaviruses and hantaviruses as well. Such diseases can all be regarded as within the clinical continuum of hemorrhagic fevers and frequently involve thrombocytopenia, coagulopathy, shock, and capillary leak syndrome. Members of both virus groups are susceptible to the antiviral drug ribavirin in vitro and in vivo.

There are certainly important differences as well. Arenaviruses are frequently transmitted vertically in the animal reservoir. Vertical transmission does not appear to occur with hantaviruses. Arenavirus disease often includes a profound neurologic component, and hepatitis is common. Hantaviruses can cause elevation of serum transaminases, but rarely is it severe, and neurologic disease is absent. In both the patient and the rodent host, antibody and neutralizing antibody responses elicited by arenaviruses are weak and arise late in infection, whereas hantaviruses elicit early and strong antibody responses (Hjelle et al., 1997; Bharadwaj et al., 2000). Treatment of some arenavirus diseases can include passive immunization using immune plasma, but the efficacy of such treatment in hantavirus infections is not known.

Research on pathogenesis is currently on a much higher technical plane for arenaviruses than for hantaviruses. An important reason is that lymphocytic choriomeningitis virus (LCMV) naturally infects the laboratory mouse *Mus musculus*, and the availability of many specialized research reagents available for that species has led to its becoming the subject of years of scrutiny as a model for the study of viral pathogenesis and immune responses (Buchmeier et al., 2007; Gonzalez et al., 2007). Furthermore, numerous animal disease models are available for arenaviruses. Fundamental studies of the immune responses to antigens, dissection of the role of the major histocompatibility complex genes in antigen presentation, the description of cytotoxic T lymphocytes (CTL), and other basic immunology studies made early and critical uses of the LCMV-mouse disease model. Investigators have used the model to map those antigenic epitopes responsible for viral clearance by class I-restricted immune responses, as have escape mutants. Both viral and host determinants of persistence, clearance, and pathogenesis of several types of diseases have been identified. Investigators have used transgenic mice in many cases to more

thoroughly evaluate the host determinants of immune response, clearance, and pathogenesis.

Some hantaviruses can be coaxed to elicit at least transient infections in hamsters or in their native rodent hosts, and recently, disease models have been described that mimic some key aspects of HCPS, albeit with several critical differences. Very little is known about the mechanisms of pathogenesis or clearance. Hantaviruses are much harder to isolate from natural sources than are arenaviruses, although for most, but by no means all, hantaviruses, an isolate is possible with prolonged efforts involving samples from multiple infected rodents (Galeno et al., 2002; Zhang et al., 2007). Once an isolate has completed its adaptation to tissue culture, such as in Vero E6 cells, it will generally propagate consistently.

Using the most rigorous species definitions, at least 12 hantavirus species and 9 arenavirus species are known or suspected to be pathogenic in humans (Table 1). The number of hantaviruses for which place names have been applied should not be used as though they each represent unique hantavirus species because many hantaviruses for which new names have been applied may not be sufficiently diverged from previously described species to be considered unique. That said, it is likely that many other authors would consider the number of distinct viral pathogens to be far higher than the 11 we recognize in Table 1. A similar situation exists for the *Arenaviridae*. In general, the taxonomies of the hantaviruses and the arenaviruses is murky and is expected to stay so for the indefinite future, due to the great complexity of the genus and the complications caused by both topographically variant ("topotypes") and truly species-defining genetic characteristics, such as those promulgated by the International Congress on the Taxonomy of Viruses (Fauquet et al., 2005). For both virus groups, Old World forms cause human diseases that are related to, but distinguishable from, those caused by New World forms. Arenaviruses from the New World have been described belonging to the Tacaribe serocomplex, a linage separated from the Old World forms. Within hantaviruses, most of the New World forms are clustered in a single clade, although the vole-borne hantaviruses such as Isla Vista and Prospect Hill viruses are part of a clade that contains European and Asian forms such as Tula virus and Puumala virus (PUUV) (Herbreteau et al., 2006).

BIOLOGY

Structure and Physical Properties

Arenavirus virions are spherical enveloped structures that generally measure between 40 and 200 nm in diameter (more typically, 110 to 130 nm) (Neuman et al., 2005). T-shaped glycoprotein spikes measuring approximately 7 to 10 nm long can be seen surrounding the surface membrane. The arenavirus particle is disrupted by detergent or solvents. When exposed to a pH of less than 5.5 or greater than 8.5, the hydrophilic head becomes detached from the remainder of the spike, and the virus can no longer bind to the cell surface receptor (Di Simone et al., 1994). The hantavirus virion is a sphere approximately 80 to 200 nm in diameter. The envelope consists of a highly structured lipid bilayer with two transmembrane glycoproteins, Gn (G1) and Gc (G2). There are three helical nucleocapsid core structures. Hantaan virus (HTNV) is inactivated by treatment at pH 5 or with 0.1% deoxycholate, formaldehyde, ether, or acetone or 70% ethanol or by storage at 37°C (Yamanishi et al., 1988).

Genome and Antigens

The single-stranded RNA genome of arenaviruses is composed of two ambisense RNA segments, large (L) and small (S), which measure approximately 7,200 and 3,400 nucleotides (nt), respectively. Each 3′ terminus has a 19-nt region of conserved bases (with 17 nt that are identical) that may hybridize to its inverted complement on the opposite end of the segment, forming panhandle structures. Both intramolecular and intermolecular complexes are formed through hybridization of the terminal inverted repeats. The L segment encodes the viral RNA-dependent RNA polymerase (or more formally, the L polymerase; 180 to 250 kDa) and a second small zinc-binding protein (Z protein; 11 kDa), that serves a role in arenavirus budding and in inhibition of host translation (Campbell Dwyer et al., 2000; Perez et al., 2004; Capul et al., 2007). The Z protein also helps abrogate the effects of the host antiviral protein interferon (IFN), perhaps through its interaction with the promyelocytic leukemia protein (Borden et al., 1998). The S segment encodes the external glycoproteins GP-1 and GP-2 as well as the 58-amino-acid (aa) stable signal peptide SSP and the core nucleoprotein antigen NP (60 to 68 kDa) (Auperin et al., 1984; Singh et al., 1987; Saunders et al., 2007). A primary function of NP is to protect the viral RNA while it is within the cell (Buchmeier, 2002). SSP, GP-1, and GP-2 are produced by posttranslational cleavage of a precursor glycoprotein, GP-C (70 to 80 kDa), by a trypsin-like protease. In addition, terminal nontemplated bases have been detected at the termini in some arenaviral genomic segments (Garcin and Kolakofsky, 1990; Raju et al., 1990; Meyer and Southern, 1994).

The mRNA for the NP antigen is complementary to the genomic RNA, whereas GP-C is translated from a genomic-sense mRNA. Similarly, the Z protein is synthesized from a genomic-sense mRNA, and the L protein is synthesized from an antigenomic-sense mRNA. GP-C is approximately 500 aa in length, with five or six N-linked glycosylation sites in GP-1 and two in GP-2. GP-1 and GP-2 are each homotetramers. GP-2 is an integral transmembrane glycoprotein and is needed for acid-dependent membrane fusion (Di Simone et al., 1994), whereas GP-1 is held in place through noncovalent interactions with GP-2 and interacts with the cell surface receptor (Borrow and Oldstone, 1992; Cao et al., 1998). NP is approximately 560 aa in length, L protein is about 2,200 aa long, and Z is about 90 aa long. NP is also subject to posttranslational modification via phosphorylation, a modification that is more abundant in persistently infected cells (Bruns et al., 1986).

The hantavirus genome is composed of three minus-sense RNA segments approximately 6,500 (L segment), 3,700 (middle [M] segment), or 1,700 to 2,100 (S segment) nt long. There are short regions (22 to 23 nt) of conserved bases on the termini that are imperfectly complementary to one another within a segment, which allows each segment to form a panhandle hairpin structure through hybridization of the 5′ to 3′ terminal inverted-repeat sequences. The trimeric nucleocapsid protein recognizes this panhandle structure, an interaction that is genus-specific, suggesting that it is conserved within the genus *Hantavirus* (Mir et al., 2006). The L segment encodes a 2,150-aa L protein, or RNA-dependent RNA polymerase. The M segment encodes a 1,140-residue envelope glycoprotein precursor. Through the cotranslational proteolytic processing of the envelope precursor in the endoplasmic reticulum, Gn (652 aa) and Gc (488 aa) are produced (Schmaljohn and Nichol, 2007), which are modified by N-linked glycosylation. The S segment encodes

TABLE 1 Pathogenic hantaviruses and arenaviruses

Virus	Distribution	Host	Disease	Comments
Hantaviruses				
Amur virus	Russia and China	*Apodemus peninsulae* (Korean field mouse)	HFRS	Highly related to HTNV
ANDV	Andes cordillera, especially Argentine and Chilean temperate forests	*Oligoryzomys longicaudatus* (long-tailed pygmy rice rat, colilargo)	HCPS	Transmissible person to person; synonymy with other pathogenic South American hantaviruses, including Araraquara, Bermejo, Castelo do Sonhos, Juquitiba, Lechiguanas, Oran, Hu39694, Andes-Central Plata, and Caño Delgadito, remains to be elucidated
Bayou virus	Eastern Texas and Louisiana; Georgia	*Oryzomys palustris* (rice rat)	HCPS	Possibly greater renal involvement than SNV
BCCV	Southern Florida	*Sigmodon hispidus* (cotton rat)	HCPS	Etiologic role in HCPS not definitively established; possibly increased renal disease
Choclo virus	Panama	*Oligoryzomys fulvescens* (fulvous pygmy rice rat)	HCPS	
DOBV	Balkan states, central Europe	*Apodemus flavicollis* (yellow-necked field mouse); *Apodemus agrarius*	HFRS, severe form	Increasingly recognized as far north as northern Germany
HTNV	Asia, specially China, Korea, and Russia	*Apodemus agrarius* (striped field mouse)	HFRS, severe	Prototype hantavirus, 5–10% case-fatality ratio; 50,000–100,000 cases/yr in China
Laguna Negra virus	Paraguay, Bolivia	*Calomys laucha* (vesper mouse)	HCPS	
New York virus	Long Island, New York	*Peromyscus leucopus* (white-footed mouse)	HCPS	On mainland, *P. leucopus* carries SNV, not New York virus; possibly synonymous with SNV
PUUV	Western Europe	*Myodes glareolus* (bank vole)	HFRS, mild (nephropathia epidemica)	Fatalities very rare despite thousands of cases in Europe
Seoul virus	Worldwide; most disease occurs in Asia	*Rattus* spp. (commensal rats)	HFRS, mild to moderate	Primary cause of HFRS in urban China
SNV	Widespread in United States and Canada, especially in western regions	*Peromyscus maniculatus* (deer mouse)	HCPS	Prototype pathogenic New World hantavirus; synonymous with eastern form known as Monongahela
Arenaviruses				
Flexal virus	Brazil	*Oryzomys* spp.	Two symptomatic laboratory infections	
GTOV	Venezuela	*Zygodontomys brevicauda* (short-tailed cane mouse)	VHF	Host originally misidentified as *Sigmodon alstoni*
JUNV	Argentina	*Calomys musculinus* (drylands vesper mouse)	AHF	Treated with convalescent-phase plasma of patients who have recovered from disease; ribavirin
LASV	Africa	*Mastomys spp*. (multimammate rats)	LAS fever (shock, hemorrhage, meningitis)	Susceptible to ribavirin
LCMV	Worldwide	*Mus musculus* (house mouse); *Mus domesticus*	Lymphocytic choriomeningitis	Prototype arenavirus
MACV	Bolivia	*Calomys* cf. *callosus* (vesper mouse)	BHF	Now rare and subject to control by intensive trapping
Sabia virus	Brazil	Unknown	Brazilian hemorrhagic fever	Three cases known; two via laboratory infection
Tacaribe virus	Trinidad and Tobago	*Artibeus* ssp. (fruit-eating bats)	Febrile syndrome	One laboratory infection with mild CNS symptoms; only known non-rodent-borne pathogenic arenavirus
WWAV	Southwestern United States	*Neotoma* spp. (woodrats)	Hemorrhagic fever	Pathogenicity must be confirmed

an RNA-binding nucleocapsid (N) core antigen, which makes up the majority of the viral core. As with the other bunyaviruses, the genomic RNAs of hantaviruses are not polyadenylated, nor are the corresponding mRNAs. One unconfirmed report has contended that the M segment of Sin Nombre virus (SNV) is indeed polyadenylated (Hutchinson et al., 1996). As with other bunyaviruses, a cap-snatching mechanism is used to initiate the synthesis of hantavirus mRNA, using primers cleaved from host cell RNAs (Patterson and Kolakofsky, 1984; Schmaljohn and Nichol, 2007).

The hantavirus envelope consists of a lattice of Gn-Gc heterodimers in a lipid bilayer. The amino terminus of each transmembrane glycoprotein is on the external surface of the membrane, and the carboxyl terminus is on the inner leaflet. The assembly of bunyavirus envelopes occurs in the *cis-* or medial-Golgi apparatus, although assembly of SNV and BCCV may occur at the plasma membrane instead (Goldsmith et al., 1995; Ravkov et al., 1997). Experiments with HTNV have shown that prior to its movement to the Golgi compartment, the nucleocapsid protein is targeted to the endoplasmic reticulum Golgi intermediate compartment, and there is a requirement of an intact endoplasmic reticulum Golgi intermediate compartment for viral replication (Ramanathan et al., 2007). There are three conserved N-linked glycosylation sites in all hantavirus Gn antigens and in one of the Gc antigens. These sites are important in protein folding and intracellular trafficking, with Gc currently regarded as the probable fusion protein of HTNV (Shi and Elliott, 2004; Zheng et al., 2007).

PATHOGENESIS

Arenaviruses readily infect a wide variety of mammals, especially rodents and bats, and a large number of experimental infection and disease models have been developed that have employed laboratory mice, guinea pigs, hamsters, and primates. Several of the animal models develop diseases that have strong similarities to the human diseases caused by arenaviruses (Peters et al., 1996). For example, clinical and histopathological findings in the common marmoset and hamster have some similarities to those reported in human cases of Lassa (LAS) fever (Sbrana et al., 2006; Carrion et al., 2007). These models have helped improve the understanding of the mechanisms of pathogenesis of arenaviruses in humans. Unlike hantaviruses, arenaviruses can also have significantly deleterious effects on their reservoir hosts (Webb et al., 1975; Childs and Peters, 1993). The nature of these effects depends upon the age of the rodent, the dose of virus, the viral serotype, the route of infection, and probably also the genetic background of the individual animal.

Arenaviruses are shed from their infected reservoir hosts in the urine, saliva, nasal secretions, and feces (Childs and Peters, 1993). In the human, infection is acquired through direct contact with infected rodents or by the inhalation of virus-contaminated aerosols (inhaling infectious rodent excreta or even when caring for rodents as pets). Organ transplantation has also been documented as a source of acquiring LCMV infection; the dosage, route, and immunosuppressed environment, considered collectively, have made infections acquired in such a manner especially deadly (Fischer et al., 2006). The main entry pathway into cells for Junin virus (JUNV) is through clathrin-mediated endocytosis (Martinez et al., 2007). South American hemorrhagic fever virus (GTOV, JUNV, and Machupo [MACV]) cellular receptors are proteins or "protein-linked entities," different from nonpathogenic arenaviruses (Rojek et al., 2006), where more than one entry pathway may be used by closely related arenaviruses related to the ability to cause human disease (Oldenburg et al., 2007). There are differences in adaptive immune response between those induced by the pathogenic LAS virus (LASV) and the nonpathogenic Mopeia virus, with activation of macrophages followed by type I IFN production more prominent in cells responding to Mopeia virus (Pannetier et al., 2004). Old World and clade C of the New World (Tacaribe complex) arenaviruses use α-dystroglycan as their entry receptor, while other Tacaribe-complex arenaviruses instead use the transferrin receptor 1, but remaining controversies in the literature suggest that multiple molecules may be needed to affect entry and early infection of permissive cells (Reignier et al., 2006; Radoshitzky et al., 2007). There is an early involvement of macrophages, and early viral replication occurs in hilar lymph nodes in some models (Kenyon et al., 1992). Arenaviruses exert an important immunosuppressive effect in animal models and in humans through necrosis of follicles and destruction of antigen-presenting cells in the lymph nodes and spleen of the host (Mims and Tosolini, 1969; Gonzalez et al., 1980). Prominent cellular destruction in vivo is a reflection of the immune response and is probably not due to the cytopathogenicity of the virus itself. While many arenaviruses will form plaques in monolayers, cytopathic effects are generally not seen.

During natural infections, arenaviruses frequently elicit only late, low-titer antibody responses, but they evoke profound T-cell responses, involving both T helper cells and CTL. Evidence based on the LCMV model suggests that slowly replicating strains elicit weaker CTL responses than those strains that more replicate more rapidly, while also influencing the duration and pathogenesis of virus persistence within the host (Bocharov et al., 2004). The CTL responses, ironically, are thought to be essential both for immunopathogenesis and for clearance and recovery in the host (Lehmann-Grube et al., 1993), consistent with some models for hantavirus pathogenesis as well. In both the mouse models and the human host, LCMV antigens are thought to elicit virus-specific $CD8^+$ T cells that are necessary and sufficient to cause neurologic disease (Byrne and Oldstone, 1984). The CTL attack and lyse the infected cells in the central nervous system (CNS), with disruption of the blood-brain barrier. In experimental models in which $CD8^+$ cells are depleted, $CD4^+$ cells may induce the same disease, but the relevance of this finding to human disease is uncertain (Muller et al., 1992). Both macrophages and dendritic cells are crucial targets for LASV but do not become activated, suggesting that they contribute to immunosuppression of LAS fever in the context of IFN's role in controlling the LASV (Baize et al., 2004; Baize et al., 2006). In fact, LCMV disables the host innate defense by inhibiting IFN (Martinez-Sobrido et al., 2006).

Arenavirus diseases of humans range from asymptomatic seroconversion through a grippe-like syndrome (fever, prostration, headache, nausea, and vomiting) to meningitis and hemorrhagic fever. Systemic and hematopoietic cell abnormalities follow the febrile state. The incubation period for LAS fever is about 5 to 21 days (Peters et al., 1996), and that for MACV is 7 to 16 days. In LCMV infection, leukopenia and thrombocytopenia, as well as elevated liver enzymes, may be seen, although in many infections there are no hematologic abnormalities (Fisher-Hoch et al., 1988). In 2000, the woodrat-borne Whitewater Arroyo virus (WWAV) was reported to have contributed to three fatal cases of a cardiopulmonary disease resembling HCPS, with lymphopenia observed

in all three patients and thrombocytopenia in two (Centers for Disease Control and Prevention, 2000). However, those results remain unconfirmed, and more studies will be necessary to determine whether WWAV is pathogenic to humans. In arenaviral disease, the febrile state is accompanied by viremia, and followed, variably and according to viral species, by CNS disease, hematologic abnormalities, and hepatitis. Hepatitis, as well as hemorrhage, is much more common and severe with LAS fever and with the South American hemorrhagic fevers than with LCMV infection, for which asymptomatic and self-limited febrile infections predominate to a very high degree over choriomeningitis, viral hemorrhagic fever, or hepatitis.

Capillary leak and widespread organ involvement accompanied by shock, adult respiratory distress syndrome, and/or hemorrhage are responsible for most deaths due to LASV and the South American hemorrhagic fever viruses. The pathological findings at necropsy, which can include petechiae and hemorrhage as well as hepatocellular and splenic necrosis, are generally not severe enough to explain the death of the patient (Child et al., 1967; Elsner et al., 1973; Walker et al., 1982). The case of transplanted patients with LCMV infection showed a systemic illness with thrombocytopenia, elevated level of aminotransferases, coagulopathy, and involvement of lung, liver, and kidney (Fischer et al., 2006). The case fatality ratios range from a few percent to as much as 34% for Bolivian hemorrhagic fever (BHF). Fetal loss is common when pregnant women become infected with LASV, although LCMV is also teratogenic, causing hydrocephalus and chorioretinitis in the developing fetus (Barton et al., 2002). All arenaviruses have some propensity to affect the human nervous system. Neonatal mice that have been infected with LCMV develop lifelong persistent infection, affecting host CNS gene expression (Kunz et al., 2006). Aseptic meningitis, coma, and seizures may be seen acutely, while LASV and possibly MACV can produce nerve deafness linked to the immune response (Cummins et al., 1990). Arenavirus infections provoke high systemic IFN-α and tumor necrosis factor alpha responses. In fact, widespread involvement by IFN-stimulated genes has been reported in the host response to LCMV, as with the West Nile flavivirus, in the CNS (Wacher et al., 2007). The presence of such mediators may partially explain the clinical features of arenavirus diseases (Levis et al., 1985; Marta et al., 1999).

Hantaviruses probably enter via the respiratory tract on particles that have been inhaled and then may make contact with cells bearing the β3 integrin entry molecule or enter by another route (Gavrilovskaya et al., 1998). Additional evidence shows that a previously unidentified 70-kDa protein may serve as a candidate receptor or alternative cellular component for interaction with HTNV (Mou et al., 2006). The virus is presumably taken up into regional lymph nodes, where it undergoes primary replication. After hematogenous dissemination, it invades vascular endothelium throughout the body, where it undergoes further cycles of replication and secondary viremia. In necropsy samples, viral antigen is detectable in endothelial cells, particularly those lining small capillaries and venules, especially in the lung, kidney, liver, lymphoid tissue, and heart (Kim et al., 1993; Zaki et al., 1995; Green et al., 1998). Rarely, some antigen is detectable in the macrophage compartment in a small minority of HCPS cases. Renal tubular epithelial cells express antigen in hemorrhagic fever with renal syndrome (HFRS) (Kim et al., 1993; Groen et al., 1996). The prodromal symptoms in HCPS, such as headache, fever, myalgias, and chills, begin after an incubation period from 11 to 32 days (although reports range from a couple of days until 46 to 51 days) (Young et al., 2000; St Jeor, 2004; Vial et al., 2006), and probably coincide with secondary viremia. A study in humans who came to autopsy shows that hantavirus infection induces a typical myocarditis with structural changes that may be responsible for myocardial depression and shock in fatal HCPS (Saggioro et al., 2007). However, it is important to remember that the great majority of North American patients who came to autopsy showed no evident pathologic changes involving the heart, suggesting that functional derangements underlie the pathophysiologic damage to the heart in patients with HCPS due to SNV.

The prodromal stage of illness usually lasts from 2 to 10 days, but 2 to 5 days is most typical. For both HFRS and HCPS, the prodromal symptoms become more severe in the 24 to 48 h immediately preceding the onset of capillary leak. Nausea, headache, backache, abdominal pain, vomiting, and/or diarrhea often ensue. Facial flushing and petechiae may develop early in the course of HFRS, whereas for HCPS, a cough may herald the onset of pulmonary disease. Immediately before the onset of capillary leak syndrome, enlarged and highly activated lymphocytes (immunoblasts) become detectable in the peripheral blood in numbers exceeding 10% of the total lymphoid series. During this time, viral antigen, expressed in the vascular endothelium, may lead to the recruitment of virus-specific CTLs to the lungs and kidneys. Autopsy studies verify the presence of immunoblasts in the interstitium of the lungs, the lymphoid organs, and the liver (Hjelle et al., 1995b; Nolte et al., 1995).

There is abundant evidence that hantavirus disease is predominantly caused by immunologic injury rather than direct viral lytic attack. The defining lesion for both HFRS and HCPS is capillary leak syndrome. For HFRS, capillary leak occurs retroperitoneally, whereas for HCPS it is confined to the pulmonary bed (Hjelle et al., 1995b). The breakdown at the level of vascular endothelial integrity is not visible microscopically but is strictly functional in nature (Hjelle et al., 1995b). The functional breakdown does not allow the egress of formed blood elements, such as red blood cells, but instead only protein is allowed to cross into the interstitium. The lack of visible lysis of endothelial cells that express hantavirus antigens can be taken as evidence against direct destruction of such cells by either virus or virus-specific CTL.

By the time symptoms appear, all patients have hantavirus-specific immunoglobulin M (IgM) antibodies against the viral N antigen. Given a diagnostic test of adequate sensitivity, essentially all have specific IgG antibodies as well (Jenison et al., 1994; Hjelle et al., 1997). Viral RNA titers are decreasing by the time of admission (Terajima et al., 1999; Xiao et al., 2006). HLA B*35-restricted CTL directed against viral N antigen in HCPS, as well as HLA Al- and B51-restricted CTL against N in HFRS, are detectable in the blood (Van Epps et al., 1999; Kilpatrick et al., 2004).

Immunoblasts (Fig. 1) are believed to be central in the pathogenesis of HCPS and probably in HFRS as well. In the blood, they commonly make up more than 20% of lymphocytes and are felt to be exchanging with those detected in the tissues at autopsy. Flow cytometric studies and in situ immunologic stains performed on autopsy samples verify that immunoblasts, despite the morphologic resemblance of some to activated plasma cells, are predominantly T cells (Nolte et al., 1995; Zaki et al., 1995). $CD8^+$ cells often predominate over $CD4^+$ cells. Macrophages also are found in the infiltrates. Using SNV and PUUV as models, some evidence has emerged that CTL-associated or $CD8^+$-T-cell-associated

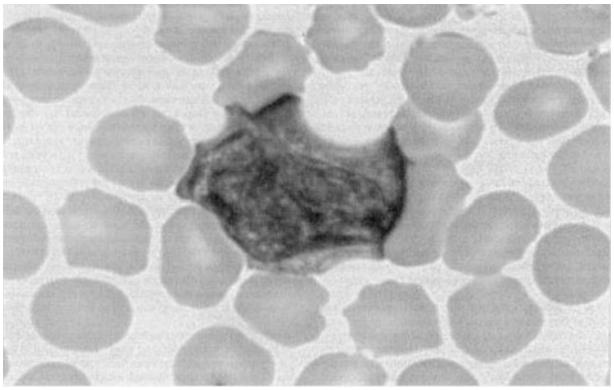

FIGURE 1 Peripheral blood smear (oil immersion) showing a typical immunoblast from a patient with acute HCPS. Note the large size (~16-μm diameter), immature chromatin, and basophilic cytoplasm. Immunoblasts are highly pleomorphic, and no single image should be taken as representative of the full spectrum of their appearance. Magnification, ×100. Photo courtesy of M. K. Foucar.

activities contribute to capillary leakage observed in patients with HCPS or HFRS (Hayasaka et al., 2007), and there is evidence for profoundly increased cytokine activity in the pulmonary bed in HCPS (Mori et al., 1999). Taken together, these findings suggest a model in which viral antigens are presented by highly activated vascular endothelial cells, resulting in recruitment of T cells to the parenchyma. T cells and resident histiocytes, as well as endothelial cells themselves, may establish a milieu in which proinflammatory cytokines induce a reversible breakdown of the barrier function of the intracellular junctions at the vascular endothelium (Fig. 2) (Puri and Rosenberg, 1989). In persistently infected mice, production of N-specific $CD8^+$ T cells remains suppressed throughout the course of infection (Taruishi et al., 2007). Under PUUV natural human infection, $CD8^+$ CTL peaked near the onset of acute response, being rare during the peak response (Tuuminen et al., 2007). SNV particles (live or inactivated) are by themselves capable of early induction of immunity (Prescott et al., 2005). This response occurs independently of IFN regulatory factor 3 and engages heretofore unrecognized pattern recognition receptors (Prescott et al., 2007).

EPIDEMIOLOGY

For both hantaviruses and arenaviruses, reservoir rodents develop chronic infections that result in transient or periodic virus shedding in urine, feces, and saliva. Infection is considered to be lifelong despite the continued presence of high titers of neutralizing and nonneutralizing antibodies in hantavirus-infected rodents.

The relationships between arenaviruses and their rodent hosts are complex, and relatively little is known about the factors that promote or inhibit arenavirus infection in wild rodent populations. Both horizontal transmission, which may be by the sexually transmitted route, and vertical transmission may occur. Horizontal transmission to immunocompetent adult animals may result in clearance, whereas neonatal infections often result in chronic viremia and shedding (Demby et al., 2001). When *Calomys* sp. mice more than 9 days of age are experimentally infected with MACV, some develop chronic viremia in the same manner as do neonates and some mount a strong antibody response and have minimal or no viremia. After a recent sexually transmitted infection, vertical transmission of MACV via milk results in chronic viremia in the pups. Dams can also transmit virus transovarially or in utero (Mims and Tosolini, 1969); if the dam has been viremic before becoming pregnant, fetal death may ensue (Childs and Peters, 1993). Fighting, including biting, is felt to be an important route for transmission of LCMV in *M. musculus* (Skinner and Knight, 1973) and for JUNV in *Calomys musculinus* (Mills et al., 1994). LCMV transmission from persistently infected mice to naïve ones occurred after direct contact of animals housed in the same cage (Ike et al., 2007).

Several different epidemiological patterns of human arenavirus infection are known. LCMV infection has a worldwide distribution, with seroprevalences ranging from approximately 1 to 10% (Peters et al., 1996). In Western Africa, thousands of cases for LAS fever have been reported each year, with antibody prevalences ranging from 7% in Guinea, to 15 to 20% in Sierra Leone and Liberia, to over 20% in Nigeria (Ogbu et al., 2007). Outbreaks of LCMV infection have occurred among people working in animal colonies or with infected animal cells or tissues (Baum et al., 1966; Biggar et al., 1975).

By comparison, LAS fever and the South American hemorrhagic fevers occur in sporadic or epidemic form in regions in which the reservoir rodent occurs. For the South American hemorrhagic fevers in particular, it is apparent that disease outbreaks are localized to discrete, small portions of the ranges of the reservoir rodents. MACV has been reported to be restricted to northeastern Bolivia, a small part of the complete range of its reservoir (*Calomys* cf. *callosus* [a *Calomys* sp. that looks like *Calomys callosus*]) (Salazar-Bravo et al., 2002b). While Venezuelan hemorrhagic fever (VHF) has thus far remained confined to a small portion of the range of *Zygodontomys brevicauda*, the area of endemicity for Argentine hemorrhagic fever (AHF) had increased steadily in the years after its initial description (Maiztegui et al., 1986). The dynamics of viral infections among host populations is complex, reflecting a combination of factors including host genetics, local extinctions of subpopulations, natural geographical barriers, and the degree and mechanism of viral pathogenesis in the host animal (Ittig and Gardenal, 2004; Polop et al., 2007).

LASV, JUNV, and MACV may be transmitted interpersonally, such as in hospital settings due to breakdowns in hygienic protocols (White, 1972; Peters et al., 1974; Fisher-Hoch et al., 1995). Male-to-female transmission, possibly sexual in some cases, has been noted for LASV and may be occurring for MACV and JUNV. LASV has been isolated from semen 6 weeks after the end of the acute stages of infection.

For hantaviruses, seroprevalences among host rodents range from about 3 to 50%, with higher prevalences in the United States generally recorded in Western states (Hjelle et al., 1995a; Mills et al., 1997; Bennett et al., 1999). In South America, high rodent seroprevalences have been reported in Paraguay, Argentina, and Chile, in some cases in the midst of rodent irruptions (Wells et al., 1997; Williams et al., 1997; Toro et al., 1998). The reservoir host does not seem to be adversely affected by the infection. The mechanisms by which rodents transmit the virus to one another have largely remained unexplored, with few exceptions (Botten et al., 2002; Padula et al., 2004). That shedding of SNV and Andes virus (ANDV) seemed to primarily involve

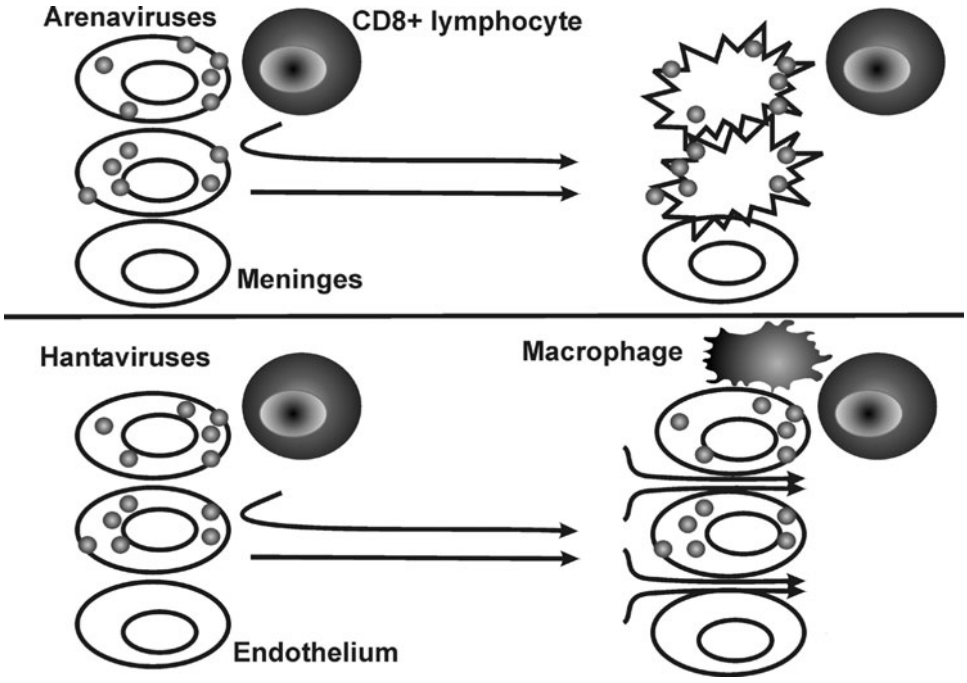

FIGURE 2 In this highly simplified diagram, the most important of the presumed immunopathological mechanisms of arenavirus infection (top) is contrasted with that of hantavirus infection (bottom). After recruitment by virus-infected cells, virus-specific $CD8^+$ cells may engage in a direct cytolytic attack and destroy the infected cells, as seen in experimental arenavirus infections. Alternatively, they may help establish a milieu that results in a functional, transient defect in the barrier function among endothelial cells, resulting in transudation of plasma from the vascular space into the interstitium. Most likely, the $CD8^+$ and/or $CD4^+$ lymphocytes must collaborate with resident tissue macrophages to elicit the defect in the endothelial barrier function. In the case of both hantaviruses and arenaviruses, it is likely that there are function defects in the barrier function of vascular endothelial cells even when cytolysis is not prominent.

saliva led Padula et al. (2004) to suggest that the main mechanism of transmission of hantaviruses among rodents would be saliva or saliva aerosols rather than feces and urine. Horizontal transmission is the predominant if not the exclusive means of transmission, with the random horizontal infection being potentially important (Iwasa et al., 2004). A potential, if undocumented, role of ectoparasites in virus transmission also has been proposed (Houck et al., 2001). Lastly, indirect transmission (even for a prolonged period of time) between rodents or from rodents to humans has been proposed for PUUV (Kallio et al., 2006). For many hantaviruses, higher seroprevalences are found in older individuals, especially in males (Douglass et al., 2007). Hantaviruses may infect rodents other than their primary reservoir hosts, especially during epizootics. Such infections also appear to be persistent, at least when the secondary host is closely related to the predominant host species (Song et al., 1995; Rawlings et al., 1996).

Humans acquire hantavirus infection by exposure to contaminated rodent excreta and/or secreta. Overwhelmingly, the evidence is that indoor exposure in poorly ventilated spaces is much riskier than outdoor activities, such as hiking, planting crops, or gardening. Vacuuming, sweeping, shaking rugs, and dusting are among the activities that aerosolize particulates that are then inhaled; descriptions of such activities that precede the first onset of symptoms by 1 to 5 weeks are extremely common among HCPS patients. It is also quite common for patients to report recent sighting of living rodents or other evidence of very recent rodent activity; thus, one may reasonably suspect that only recently produced rodent excreta are highly infectious to humans. The importance of peridomestic settings as a major area where exposure occurs has been emphasized, but there have been systematic biases in the methods by which patients are surveyed that have likely overstated the degree to which peridomestic exposures predominate (Kuenzi et al., 2001; Douglass et al., 2006). Recreational and occupational exposures also have been recognized when sought (Hjelle et al., 1996; Jay et al., 1996; Torres-Perez et al., 2004).

Hantavirus infections occur somewhat more frequently in men than women; male/female infection ratios range from 1:1 to more than 4:1 (Niklasson and Leduc, 1987; Niklasson et al., 1987; Ruo et al., 1994; Ferrer et al., 1998). Hantaviruses rarely infect children under 12, except for ANDV in Argentina and Chile (Pini et al., 1998). However, children and adolescents (10 to 16 years old) have a clinical course and case fatality ratio similar to those of adults (Ramos et al., 2001). Most likely, all racial and ethnic groups are susceptible to hantavirus infection and disease. In South America, no differences in seroprevalences were found between indigenous Chileans and those with Hispanic ancestry (Castillo et al., 2002; Tager et al., 2003).

However, there may be associations between immune response gene genotypes and disease severity: investigators found that patients with mild disease due to ANDV were enriched in the HLA-DRB 1*15 allele, while a similar enrichment of the class I marker HAL-B*08 among those with more severe disease was also uncovered (Ferrer et al., 2007), similar to the observations reported for PUUV (Mustonen et al., 1998). In the United States, native Indian groups are overrepresented among HCPS patients. The overrepresentation of Indians can be attributed to their more frequent involvement in rural, agrarian lifestyles compared to members of the majority culture, but the marked higher incidence of HCPS among Navajo Indians compared with those among nearby Pueblo Indian tribes, such as the Hopi, suggests that genetic variations could also contribute to susceptibility to infection or disease.

Interpersonal transmission of ANDV is a well-established phenomenon in Argentina and Chile (Enria et al., 1996; Padula et al., 1998; Ferres et al., 2007). Clustered cases of human infection have been noted for ANDV in both countries (Martinez et al., 2005; Ferres et al., 2007; Lazaro et al., 2007). For the North American and Eurasian hantaviruses, common-source exposures to rodent-infested houses, buildings, and vehicles have been judged to be responsible for case clusters. Both nosocomial and intrahousehold transmission have been documented, although not at equal levels of rigor (Enria et al., 1996; Pinna et al., 2004). Nosocomial transmission has been reported in Argentina (Castillo et al., 2004). The mechanism of interpersonal transmission is not well established, and the biological basis still remains to be characterized, although recent studies demonstrate that protracted, relatively intimate interpersonal contact is required and that sexual partners share a 20-fold greater risk than other family members (Martinez et al., 2005; Ferres et al., 2007). No such transmission has been demonstrated for any other hantavirus.

DIAGNOSIS

Diagnosis of acute infection with arenaviruses is often problematic because specific antibody responses to arenavirus infections are often delayed until days to weeks after the illness has run its course. IgM and IgG antibodies may be detected by indirect immunofluorescence assay using infected cells as a substrate or by enzyme-linked immunofluorescence assay, Western blotting, or other immunoblot assay using recombinant antigens or viral lysates. Detection of live LASV by indirect immunofluorescence assay is possible about 5 to 7 days after the start of culture using peripheral blood from patients, but antigen testing or testing for specific IgM antibodies gives a faster result (Buchmeier et al., 2007). Neutralizing-antibody responses may be detected but are not diagnostically useful in the acute-care setting. Assays for circulating viral antigen, which use sandwich immunoassays, are sensitive for diagnosis of acute LAS fever, at least in fatal cases (Peters et al., 1996). A highly sensitive and specific reverse enzyme-linked immunosorbent assay (ELISA) methodology for IgG and IgM antibodies for detecting LASV also has been described (Emmerich et al., 2006). A universal reverse transcriptase (RT)-PCR assay has been developed for detection of New World arenaviruses, together with a real-time RT-PCR for JUNV and GTOV based on fluorescence resonance energy transfer probes (Vieth et al., 2005). Unfortunately, such tests are not widely available in the acute-care setting. Virus isolation is possible either in susceptible mice or in tissue culture, but it is not practical due to its inconsistent sensitivity and the high biohazard (biosafety level 4) associated with propagation of all of the natural human arenavirus pathogens other than LCMV. For these reasons, tests for viral genetic material in inactivated patient blood have been increasingly favored in the diagnosis of acute arenavirus infection (Lunkenheimer et al., 1990; Lozano et al., 1995). Unfortunately, such technology is not readily available in many of the remote, impoverished regions of the world, such as western Africa or northern Bolivia, where arenavirus diseases occur. However, a recombinant nucleoprotein-based diagnostic system has been developed to diagnose LASV without requiring infectious virus (Saijo et al., 2007). It also was noted that JUNV RNA could be detected in some febrile patients who did not seroconvert to JUNV, suggesting the possibility that JUNV may cause infection without seroconversion in some cases. Because of its distribution, LAS fever must be differentiated from other febrile diseases like Ebola fever, yellow fever, malaria, diphtheria, legionella, and Congo hemorrhagic fever. Useful clinical predictors of LAS fever are fever, pharyngitis, retrosternal pain, and proteinuria (McCormick et al., 1987).

By comparison to arenaviruses, specific serologic diagnosis of acute hantavirus infection is straightforward. Given a test of sufficient sensitivity, hantavirus antibodies of both the IgM and IgG classes are readily detectable in virtually all patients from the onset of symptoms (Hjelle et al., 1994a, 1997; Jenison et al., 1994). Tests using recombinant antigens that have been affixed to a membrane (Western blotting or strip immunoblot assay) appear to be more sensitive than ELISAs, especially in the IgG format (Hjelle et al., 1997). For the more severe hantavirus diseases, such as HCPS and the Asian forms of HFRS, viral RNA is readily detectable by RT-PCR, using peripheral blood mononuclear cells as the source of RNA (Hjelle et al., 1994c). Simple, specific, and rapid immunochromatographic IgM-antibody tests have been used for diagnosing PUUV, HTNV, and Dobrava-Belgrade virus (DOBV) (Hujakka et al., 2003) and can detect antibodies to ANDV though with modest sensitivity of a bit over 90% (Navarrete et al., 2007). Recently, new methods have been developed that improve diagnosis in the New World and Old World hantaviruses, for example, IgG and IgA ELISAs based on yeast-expressed nucleocapsid proteins of PUUV and DOBV (Meisel et al., 2006). A real-time RT-PCR was also developed as a useful method for diagnosis of PUUV viremia and for detecting PUUV RNA at early time points, even before the appearance of IgM antibodies (Evander et al., 2007). Serological assays based on ANDV yeast-expressed nucleocapsid protein have been shown to be sensitive for ANDV infection and capable of facilitating its early diagnosis (Schmidt et al., 2006). Hantavirus antigens may also be readily detected in paraffin-embedded tissues of patients at necropsy, but because serologic and blood RT-PCR tests are available, tissue biopsy is not necessary to diagnose infection in living patients (Zaki et al., 1995; Green et al., 1998). Viral RNA is readily detectable by RT-PCR using frozen tissues and also may be detected in paraffin-embedded tissues (Nichol et al., 1993; Hjelle et al., 1994b; Schwarz et al., 1995; Heiske et al., 1999).

Specific diagnostic techniques are generally available only in regional laboratories. Even given the relatively brief (several hours) turnaround times for antibody tests offered in some laboratories, shipping of samples may impose an obligatory 24- to 30-h delay in diagnosis. Such delay may be unacceptable for diagnosis of HCPS and some cases of HFRS. For HCPS, clinical algorithms may be used to ascertain more quickly whether the disease is progressing as expected

TABLE 2 Clinical and laboratory findings especially helpful in clinical diagnosis of HCPS and HFRS

Characteristic	Sensitivity	Specificity	Comments
Prodrome of fever, headache, myalgias with or without nausea and vomiting	100%	Low	Nausea and vomiting are common but not universal. Muscle aches may be severe, even limiting, and tend to involve lower muscle groups in thighs and buttocks.
Cough	Moderate	Low	Predicts pulmonary edema.
Elevated lactate dehydrogenase; liver function test abnormalities	High	Modest	Aspartate transaminase and alanine aminotransferase elevation may occur relatively late in disease. Lactate dehydrogenase elevation occurs early but is not always profound.
Left shift	High; nearly 100%	Low	Not "bandemia" but must include granulocytes at myelocyte or earlier stage to be specific. Granulocytes are rarely highly activated.
Thrombocytopenia	100%, but is, on rare occasions, delayed for 1–2 days	Moderate	A key laboratory abnormality that can often decisively exclude HCPS when absent. Sensitivity with some forms of HFRS is less impressive.
Elevated serum lactate level	Modest	Modest	Predicts severe disease.
Flushing, petechiae	Moderate (HFRS only)	Moderate	Common for HFRS caused by HTNV. Petechiae may occur in palate, pharynx, or conjunctivae.
Conjunctival injection, photophobia, ophthalmic pain, glaucoma	Moderate (HFRS only)	Moderate	HFRS only.
Renal insufficiency, proteinuria	Moderate (HFRS >> HCPS)	Moderate in HFRS, low in HPCS	Common in HTNV-associated HFRS; much less so in PUUV HFRS or in HCPS.
Bilateral interstitial infiltrates	100% in HCPS; low in HFRS	Moderate	Some SNV infections are now recognized without pulmonary involvement. HTNV-associated HFRS may result in interstitial infiltrates in up to 20% of cases.

for HCPS. Thrombocytopenia is nearly universal upon presentation, even at the earliest stages. Thrombocytopenia progresses very rapidly, often with declines of 50,000 platelets/μl or more in as little as 12 h. Serial platelet counts may be monitored pending the results of specific antibody (IgG and IgM) tests, which provide the definitive diagnosis, but in no case should ordering of a serologic test be delayed so that clinical or hematologic parameters can be evaluated over time, since that can delay the diagnosis and increase the chance of a poor outcome. For HCPS, manual peripheral blood smears may be evaluated for the presence of immature, nonactivated granulocytes and the appearance of lymphocytes with the characteristic morphologic features (Fig. 1) (Koster et al., 2001; Mertz et al., 2006). Such approaches may be equally useful for some forms of HFRS, but laboratory abnormalities in milder diseases such as HFRS may be less extreme. Given evidence that the disease is progressing as expected for HCPS or severe HFRS, it is advisable that the patient be transported to a facility with advanced tertiary-care capabilities. Abnormalities that are judged to be especially sensitive and specific for early recognition of HCPS (and, to a lesser extent, for HFRS) are outlined in Table 2.

PREVENTION

One may reduce one's risk of exposure to hantaviruses and arenaviruses by reducing contact with wild rodents. By keeping cooking and eating areas clean, containing garbage, sealing holes in buildings, and clearing trash from the outside of buildings, one may make the home less attractive to rodents. Trapping or poisoning may be used to reduce rodent infestations. For LCMV, serologic monitoring has been suggested for people who are involved in working with and transferring laboratory rodents between institutions (Ike et al., 2007). However, rodent control sometimes seems to be impractical, mainly in cases where the virus reservoir preferentially inhabits wild and/or open areas (e.g., countryside, forest, or pampas). Prevention efforts should be instituted even in those areas where only temporally remote or no human cases have been reported but where the rodent reservoir is distributed (Sinclair et al., 2007).

Indoor spaces that have become infested with rodents represent a significant risk, and their cleaning must be handled carefully. The space should be ventilated extensively in advance. Rodent droppings should be thoroughly wetted with detergent or 10% hypochlorite solution before cleaning. Even at that point, every effort should be made to avoid aerosolizing dust, such as by avoiding the use of leaky handheld vacuum cleaners or the brisk sweeping of dust in closed spaces. Using soapy liquids extensively during cleaning helps prevent any residual live virus from becoming airborne. One should wear gloves and, when available, HEPA masks during cleaning activities. Rodent carcasses should be disposed of in sealed plastic bags after spraying them with detergent or bleach. Insecticide spray may also be applied to

the rodent carcass in areas in which plague occurs. Prevention of AHF by vaccination with the Candid 1 vaccine has been recommended for children under age 15 in those areas where the disease is considered to be endemic (Feuillade and Enria, 2005).

A replication-competent vaccine against LASV based on attenuated recombinant vesicular stomatitis virus has been developed, and testing in nonhuman primates showed promising results, but regulatory issues remain to be overcome (Geisbert et al., 2005). New epitopes also have been identified capable of inducing $CD8^+$ T cells, protecting mice against LASV and LCMV (Botten et al., 2006; Botten et al., 2007). Hantavax, a mouse brain-derived, formalin-inactivated vaccine has been used in humans, although a cell culture-based vaccine seems to provide more effective immunity against the HFRS in Asia (Choi et al., 2003). In addition, a chimeric plasmid (heat shock protein 70-based HTNV S DNA) induced both humoral and cellular immune responses specific for HTNV NP, making the plasmid a candidate vaccine for HTNV infection (Li et al., 2007).

THERAPY

Arenaviruses, such as LASV and JUNV, are quite sensitive to the broad-spectrum antiviral drug ribavirin in vitro and in vivo, and intravenous ribavirin has been used during outbreaks of arenavirus disease (McCormick et al., 1987; Enria and Maiztegui, 1994). Unfortunately, ribavirin is not always available. Convalescent-phase plasma obtained from patients who have recovered from JUNV contains neutralizing antibodies. Depending upon the neutralizing-antibody titer, convalescent-phase plasma may reduce the mortality of JUNV infection from 20 to 30% to 1 to 2%. A minority of patients treated with convalescent-phase plasma may return with a late neurologic syndrome, but the neurologic syndrome is usually self-limited and full recovery is the rule. The pyrazine derivative, T-705, has demonstrated antiviral activity against some RNA viruses (including influenza virus), with experimental studies suggesting it as a possible alternative for treatment of arenaviral (e.g., Junin, Pichinde, Tacaribe) and bunyaviral (e.g., La Crosse, Rift Valley fever, sandfly fever) infections (Gowen et al., 2007). Newer therapeutic approaches such as synthetic oligodeoxynucleotides that contain unmethylated CpG motifs that activate the innate immune system, have shown a protective effect in neonatal mice from a neurotropic viral infection such as that produced by Tacaribe virus (Pedras-Vasconcelos et al., 2006).

Treatment of hantavirus infections is largely supportive, although ribavirin could assume an important place in the treatment of HFRS due to HTNV in China (Huggins et al., 1991). In vivo studies of deer mice have shown that ribavirin, human convalescent-phase plasma, and anti-β3 integrin antibody inhibit seroconversion by SNV (Medina et al., 2007). For HTNV, PUUV, Seoul virus, ANDV, and SNV, vaccines based on cDNAs of genes from M and S viral segments have been found capable of eliciting antibody responses that neutralize viruses (Bharadwaj et al., 2002; Hooper et al., 2006; Lindkvist et al., 2007). For HCPS, early placement of the patient in the intensive care unit is thought to be critical in reducing mortality. Early recognition during the thrombocytopenic prodrome phase and transport to intensive care improves survival, as can limited fluid replacement, early inotropic therapy, and mechanical ventilation (Chang et al., 2007). For patients with severe cardiopulmonary manifestations that are predictive of demise (a cardiac index of <2 and serum lactate at >4 mg/dl), extracorporeal membrane oxygenation has been used and has prevented death in several gravely ill patients. For HFRS, there is a generally less-severe spectrum of disease. Nephropathia epidemica due to PUUV, the predominant European form of HFRS, is usually mild and can often be treated in the outpatient setting. Physicians in the region of endemicity often quickly recognize HFRS due to HTNV, a much more severe disease, during the prodrome period. Such early recognition can permit timely intervention with ribavirin.

A placebo-controlled, double-blind trial of ribavirin was undertaken from 1996 through 2001 in the United States and Canada for HCPS treatment (Mertz et al., 2004). Ribavirin seemed not to be effective in the treatment of HCPS in the cardiopulmonary stage, although more study could be needed to assess its efficacy in earlier stages of infection. However, hyperimmune serum might represent a future therapy because survival seems to be correlated with higher neutralizing antibody titers at admission (Chang et al., 2007). Treatment alternatives, such as a controlled trial of methylprednisolone therapy conducted for HCPS in Chile (Mertz et al., 2006), are still in development.

FUTURE

Preventing morbidity and mortality from arenavirus and hantavirus infections is a daunting task fraught with many logistical and economic difficulties. However, many important inroads have been made. While LAS fever is a significant cause of morbidity and mortality in Western Africa, preventing and treating LAS fever is rendered very difficult by the limited economic resources available to the affected populations. BHF has been controlled by rapid and aggressive rodent-trapping programs, and AHF is potentially controllable by the Candid 1 vaccine (Maiztegui et al., 1998). The mortality of HFRS in China has been reduced by the limited use of ribavirin, hantavirus vaccines, and improved early recognition of the syndrome. While education has undoubtedly had important but unquantifiable effects, less progress has been made in the control of viral hemorrhagic fever, nephropathia epidemica, or HCPS. For HCPS, which affects the wealthiest nations of North and South America, education has been inadequate to significantly reduce the mortality of the disease, whether in outbreak or sporadic forms.

For LAS fever, BHF, VHF, HFRS, and HCPS, vaccines represent the most probable route toward control of morbidity and mortality. Several recent studies seem to promise the development of new vaccines both for Old and New World virus diseases. Commercial interest in vaccine development is generally slight, however, either because the market is considered to be too small or because the affected populations do not have the economic resources to pay for the vaccine (Hjelle, 2002). It is nevertheless important for scientists to proceed apace with developing such vaccines and demonstrating their efficacy in animal and human systems so that their existence can be highlighted in the commercial and political arenas in which their ultimate fate will be decided. Modern tools for predicting disease outbreaks have been increasingly developed, which should be in more fluent knowledge for the local health services.

CONCLUSIONS

In addition to their considerable economic impact in destruction of crops and food stores, rodents carry a variety of diseases that afflict humans. The viral diseases carried by

rodents include the hemorrhagic fevers caused by arenaviruses (family *Arenaviridae)* and hantaviruses (family *Bunyaviridae)*. These disparate virus groups, related at a most distant level phylogenetically, have many fascinating similarities to one another and equally fascinating differences. Arenaviruses and hantaviruses affect thousands of people every year, with a high fatality rate in some areas. By striking rural, often impoverished populations with severe diseases, frequently in epidemic form, they present unique challenges to the world's public health infrastructure. Modern tools for characterization, diagnosis, treatment, and prevention of diseases caused by these pathogenic viruses have been developed for the health and science community, but there is still much to be done.

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APPENDICES

III

Virology Services Offered by the Federal Reference Laboratories at the Centers for Disease Control and Prevention

BRIAN W. J. MAHY

APPENDIX 1

Strong partnerships and collaborations among local, state, and federal laboratories provide the foundation for a successful national program to prevent and control viral infectious diseases. On the international level, laboratory partnerships have become increasingly critical as new diseases and microbes continue to proliferate and cross borders. Over the past decade, the Centers for Disease Control and Prevention (CDC) has worked to strengthen laboratory capacity to respond to infectious disease threats. At CDC, multiple laboratories provide reference testing services in microbiology, hematology, histopathology, and immunology. These laboratories are part of several divisions that are housed within four national centers: the National Center for Immunization and Respiratory Diseases (NCIRD); the National Center for Human Immunodeficiency Virus (HIV)/AIDS, Viral Hepatitis, Sexually Transmitted Disease, and Tuberculosis Prevention (NCHHSTP); The National Center for Preparedness, Detection, and Control of Infectious Diseases (NCPDCID); and the National Center for Zoonotic, Vector-borne, and Enteric Diseases (NCZVED). These four National Centers form CDC's Coordinating Center for Infectious Diseases (CCID).

In addition, CDC has established the Laboratory Response Network (LRN), a multilevel network that links more than 150 public health, veterinary, military, food testing, and environmental laboratories in the United States, Canada, the United Kingdom, and Australia to ensure prompt detection and rapid response to naturally occurring or intentionally caused disease threats. The LRN laboratories are designated as either national, reference, or sentinel laboratories depending on their testing and biosafety level capacities. The varying specialties and capacity levels of these laboratories enable them to provide the highest level of expertise to quickly recognize, rule out, confirm, or definitively characterize infectious pathogens.

SPECIMEN SUBMISSION

State health departments and other federal agencies may submit specimens for reference testing to CDC. All specimen submissions first require approval by individual state health departments. CDC laboratories also work with the World Health Organization and ministries of health of other nations to perform testing on viral isolates and clinical specimens. Examples of specimens received for testing include:

- Cultures, serum, or cerebrospinal fluid samples, transudates, exudates, tissues, or histologic specimens from patients suspected of having an unusual infectious disease and/or other kinds of specimens (e.g., vectors, foods, liquids) that aid in the diagnosis of life-threatening, unusual, or exotic infectious diseases.
- Cultures or serum specimens obtained from patients with infectious diseases that occur only sporadically or from patients involved in outbreaks of diseases caused by organisms for which satisfactory diagnostic reagents are not commercially or widely available.
- Organisms suspected of being unusual pathogens or that are associated with hospital-acquired infections.
- Specimens sent for confirmation of quality assurance for test performance.
- Clinically important specimens that showed atypical, aberrant, or difficult to interpret results at state laboratories.
- Arthropod and vertebrate specimens necessary for confirmation of zoonotic diseases.

Information on specimen collection and shipping requirements along with specimen submission forms are available on CDC's website, as listed in Table 1. Additional information for testing and presumptive agent identification for select agents is available at CDC's emergency preparedness and response website (http://www.bt.cdc.gov/labissues).

TABLE 1 Virology laboratories and services at CDC

Center	Division and branch(es) or program(s)	Website and/or contact information for laboratories
NCIRD	Influenza Division Immunology and Pathogenesis Branch Molecular Virology and Vaccines Branch Virus Surveillance and Diagnosis Branch	http://www.cdc.gov/flu/professionals/diagnosis/index.htm Website includes: Role of laboratory diagnosis Influenza symptoms and laboratory diagnostic procedures Rapid diagnostic testing: information for health care professionals Rapid diagnostic testing: information for clinical laboratory directors Interim guidance for influenza diagnostic testing during the 2006–2007 influenza season
	Division of Viral Diseases Gastroenteritis and Respiratory Virus Laboratory Branch Polio and Picornavirus Laboratory Branch Measles, Mumps, Rubella, and Herpesvirus Laboratory Branch	Division of Viral Diseases inquiries (800) CDC-INFO [(800) 232-4636] (888) 232-6348 (TTY) cdcinfo@cdc.gov
NCZVED	Division of Vector-Borne Infectious Diseases (DVBID)	Instructions for sending diagnostic specimens to the DVBID Arbovirus Diagnostic Laboratory: http://www.cdc.gov/ncidod/dvbid/misc/arboviral_shipping.htm
	Arbovirus Disease Branch	Centers for Disease Control and Prevention DVBID 3150 Rampart Road Fort Collins, CO 80521
	Dengue Branch	Dengue Branch Centers for Disease Control and Prevention 1324 Cañada Street San Juan, Puerto Rico 00920-3860 Phone: (787) 706-2399 Fax: (787) 706-2496
	Division of Viral and Rickettsial Diseases Rickettsial Zoonoses Branch Poxvirus and Rabies Branch Special Pathogens Branch Infectious Diseases Pathology	Specimen submission information: http://www.cdc.gov/ncidod/dvrd/spb/mnpages/specimen.htm
NCHHSTP	Division of Global AIDS International Laboratory	http://www.cdc.gov/globalaids/default.html
	Division of HIV/AIDS Prevention—Surveillance and Epidemiology HIV Laboratory	http://www.cdc.gov/hiv/
	Division of Sexually Transmitted Diseases Laboratory Reference and Research	http://www.cdc.gov/std/
	Division of Viral Hepatitis Laboratory Branch	Viral hepatitis serology online training: http://www.cdc.gov/hepatitis/Resources/Professionals/Training/SerologyStart.htm
NCPDCID	Division of Emerging Infections and Surveillance Services Arctic Investigations Program	http://www.cdc.gov/ncidod/aip/research/research.html
	Division of Bioterrorism Preparedness and Response Laboratory Response Branch	Includes the LRN: http://www.bt.cdc.gov/lrn/
	Division of Scientific Resources Animal Resources Branch Specimen Management Branch	http://www.cdc.gov/ncidod/srp/index.html Includes information on specimen shipping/packing, importing, reference testing; and drug service
	Division of Laboratory Systems Laboratory Systems Development Laboratory Practice Standards Laboratory Practice Evaluation and Genomics	Information on best practices, CLIA, training: http://wwwn.cdc.gov/dls/default.aspx International Laboratory-Related Resource and Activity Directory: http://wwwn.cdc.gov/dls/ila/default.aspx

State Public Health Laboratory Virology Services

ROSEMARY HUMES

APPENDIX 2

Public health laboratories provide services essential to key sectors of the public health infrastructure—disease control and prevention, maternal and child health, environmental health, epidemiology, and emergency preparedness and response. These services support both population-based public health practices and primary health care needs. While all state public health laboratories provide testing for a wide range of infectious diseases, test menus vary depending on state resources and the needs of individual constituencies.

As described in *Core Functions and Capabilities of State Public Health Laboratories* (Association of Public Health Laboratories, 2000), all state public health laboratories should have the ability to (i) provide testing that supports the rapid recognition and prevention of the spread of communicable diseases, which includes isolating and identifying the causative agent, determining the source of infection, identifying carriers, and locating sources of infection in the environment; (ii) serve as a center of expertise for the detection and identification of biologic agents of importance in human disease; (iii) provide specialized tests for detecting and identifying low-incidence, high-risk diseases and newly emerging pathogens and for monitoring epidemiologic trends; and (iv) perform tests to meet specific program needs of public health agencies, such as human immunodeficiency virus (HIV) and sexually transmitted disease prevention programs.

Since 2001, the public health laboratories' slate of responsibilities has grown significantly, reflecting the nation's changing health and security environment. Beginning in fiscal year 2001, the Centers for Disease Control and Prevention (CDC) Public Health Preparedness and Response for Bioterrorism cooperative agreement has funded activities to strengthen the public health laboratory system for efficient and effective response to potential acts of bioterrorism, infectious disease outbreaks, and related emergencies. As a result of this funding, all state public health laboratories now have the appropriate equipment and expertise to use molecular methods such as PCR and other nucleic acid tests to quickly identify and characterize infectious agents.

Public health laboratories continue to serve as the backbone of the Laboratory Response Network (LRN). Founded in 1999 by the CDC, the Association of Public Health Laboratories (APHL), and the Federal Bureau of Investigation, the LRN is the nation's premier laboratory system for detecting, confirming, and reporting agents of biological terrorism in all matrices and agents of chemical terrorism in clinical specimens. Public health laboratories serve as LRN reference level laboratories, producing high-confidence test results that are the basis for threat analysis and intervention by both public health and law enforcement authorities. Most hospital and commercial reference laboratories have been classified as sentinel (formerly level A) laboratories. The primary role of sentinel laboratories is to "raise suspicion" when rule-out testing indicates a targeted agent may be present in a clinical sample and to promptly refer suspicious isolates and specimens to an LRN reference laboratory for confirmatory testing (Humes and Snyder, 2007). Public health LRN laboratories also provide training and conduct outreach to sentinel clinical laboratories.

Accurate and timely laboratory analyses are critical to identifying, tracking, and limiting public health threats and ultimately reducing rates of preventable morbidity and mortality. The state public health laboratories have expanded their leadership role to ensure that essential and state-of-the-art laboratory services are provided and that clinical laboratories that perform public health testing on reportable infectious diseases submit results to the public health surveillance system in compliance with jurisdictional requirements. To enhance disease detection, response, and control, state public health laboratories are endeavoring to build laboratory systems that maintain and enhance working relationships with all partners involved in public health surveillance and testing, including sentinel clinical laboratories, local public health laboratories, veterinary, agricultural, food safety, university, and military laboratories as well as local law enforcement and the Federal Bureau of Investigation.

Within this context, some of the functions and viral diagnostic services available in state public health laboratories are described here. This information was obtained through data from several surveys conducted by the APHL and from individual state laboratory service directories. Issue briefs summarizing public health laboratory capacity for a wide range of services are published on the APHL website (www.aphl.org).

SCOPE OF VIROLOGIC SERVICES

The scope of virologic testing provided in each state public health laboratory is determined by multiple factors, including the availability of routine virology services in the

private sector within the jurisdiction, state epidemiologic surveillance priorities, and budget. As new technologies have increasingly expanded the ability of hospital and commercial laboratories to provide routine virology services, some states have shifted their focus to disease surveillance priorities and detection of emerging pathogens. An overview of the services available in each state laboratory is provided in Table 1. A completely accurate listing of specific services is not possible here, as the scope of services changes periodically. It is recommended that you refer to your state laboratory to determine the extent of services offered. The addresses, phone and fax numbers, and websites for the state laboratories, as of April 2007, are provided in Table 2. Forty-six (92%) of the state laboratories currently have their own websites.

Depending on the jurisdiction, detection of viruses may involve viral isolation using traditional cell culture and shell vials, direct detection methods such as immunofluorescence, enzyme immunoassays, electron microscopy, and nucleic acid amplification. All state public health laboratories perform virus isolation for influenza viruses as part of the United States-World Health Organization Collaborating Laboratories network and National Respiratory and Enteric Virus Surveillance System. In this role, it is critical that state public health laboratories receive a subset of early, mid-, and late-season isolates from the clinical laboratories in their jurisdiction. Many also provide culture-based testing for other respiratory viruses (e.g., adenoviruses, parainfluenza virus types 1, 2, and 3, and respiratory syncytial virus), enteroviruses, and herpesviruses. Forty-seven states have at least one biosafety level 3 suite within their laboratory.

All laboratories rely on well-validated testing methodology to provide accurate and timely test results. In addition, the public health laboratories must be able to identify health problems, including clusters of disease outbreaks and emerging infectious diseases. For this, state-of-the-art diagnostics are the best tool the laboratories have. The CDC plays a key role in assisting public health laboratories in building and enhancing molecular diagnostic capability and capacity. Assays developed at the CDC and deployed to public health laboratories through the LRN and APHL have been used to rapidly respond to West Nile virus, severe acute respiratory syndrome, and monkeypox as well as to assist in confirming adverse events during the smallpox vaccination program. As part of the CDC's efforts to prepare for the possible emergence of a new influenza strain or entry into the United States of highly pathogenic avian influenza H5N1, all state public health laboratories are able to rapidly detect and subtype influenza directly from clinical samples or from viral isolates using molecular methods. Molecular amplification assays also are used by many state public health laboratories for qualitative detection of norovirus, arboviruses, rabies, enteroviruses, herpesviruses, including varicella, and *Chlamydia trachomatis* directly from clinical specimens. As a component of bioterrorism preparedness, all state laboratories have the capability to detect orthopox viruses from clinical samples or virus culture, and approximately half of the states meet CDC-specific criteria to perform variola-specific molecular assays. Quantitative assays for monitoring HIV and/or hepatitis C virus infection is offered in some laboratories.

The types of serologic assays provided in state public health laboratories vary, but many perform serologic testing for HIV and hepatitis A, B, and C and immunoglobulin M and immunoglobulin G assays for vaccine-preventable diseases, including varicella, measles, mumps, and rubella viruses, arboviruses (including West Nile virus), and hantavirus.

Rabies testing is a unique function of public health laboratories, as these services are generally not available in clinical or private laboratories. Rabies testing is performed in 44 state public health laboratories.

All state public health laboratories have the ability to access expertise at the CDC and forward specimens for supplemental or confirmatory testing when needed.

SUBMISSION OF SPECIMENS

All states and U.S. territories have laboratories that accept specimens for the diagnosis of viral diseases (Table 1). Submission of specimens may be made directly from the requesting physician or clinical or commercial laboratory or via local public health laboratories. Each laboratory has its own set of requirements for the processing, shipping, and types of specimens that are acceptable for the detection, identification, and/or characterization of particular viruses. These requirements are described in written and electronic directories of service provided by the appropriate state authority in each jurisdiction. During an outbreak, many public health laboratories will limit the number of specimens that they accept of disease detection, confirmation, or agent characterization (i.e., norovirus outbreaks, seasonal influenza subtyping). Additional information regarding specimen submission requirements should be obtained by contacting the laboratory directly. Some generally accepted requirements include:

1. Patient demographic information, relevant clinical history and symptoms, type of specimen, collection date, virus(es) for which the specimens are to be tested, ordering physician, and appropriate contact information for result reporting.
2. Serologic testing for antiviral antibodies generally requires simultaneous submission of acute- and convalescent-phase sera, except in special cases, such as emerging disease detection or studies for immune status of vaccine-preventable diseases, such as measles, mumps, or rubella.

Due to declining state budgets, a growing number of public health laboratories have established fees for primary diagnostic testing. As a general rule, epidemiologic surveillance testing, such as strain characterization, are conducted at no charge to the submitter. Some public health laboratories have established courier services to expedite the transport of specimens from hospitals and clinical and local public health laboratories. Cultures and clinical specimens known or suspected to contain infectious substances must be packaged according to domestic and international regulations on dangerous goods and infectious substances promulgated by the U.S. Department of Transportation, U.S. Postal Service, the International Air Transport Association, and the Canadian Transportation of Dangerous Goods Regulations.

RESULT REPORTING

While public health informatics has become the top agenda item of many government agencies and private institutions, unfortunately, there is wide disparity in the ability of public health laboratories to share their data electronically. There are two major elements of a public health laboratory information management system: one is the application that supports the daily work and functions of the laboratory to

produce relevant disease data for public health purposes, and the other piece allows the electronic communication of patient test orders and test results between the laboratory and its private, local, state, and federal partners. While 85% of state public health laboratories have a laboratory information management system in place, many lack the capability to readily exchange patient results with the clinical laboratory community electronically. The lack of interoperable laboratory systems is well recognized, and work is ongoing to address the gaps in technology and funding through the creation of a variety of initiatives, programs, and networks.

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TABLE 1 Virology services available in state and territorial public health laboratories[a]

State or territory	Viral serology	Virus isolation	NAAT[b]	Influenza surveillance	Arbovirus serology	Rabies detection[c]	Norovirus PCR	HIV serology	Hepatitis serology (A/B/C)	Chlamydia testing
Alabama	Y	N	Y	Y	Y	Y	N	Y	N/Y/Y	Y
Alaska	Y	Y	Y	Y	N	Y	Y	Y	Y/Y/Y	Y
Arizona	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Arkansas	Y	N	Y	Y	Y	N	N	Y	Y/Y/Y	Y
California	Y	Y	Y	Y	Y	Y	Y	Y	N/N//N	Y
Colorado	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Connecticut	Y	Y	Y	Y	Y	Y	Y	Y	N/Y/Y	Y
Delaware	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/N	Y
District of Columbia	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/N	Y
Florida	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Georgia	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Hawaii	Y	Y	Y	Y	N	Y	Y	Y	N/N/N	Y
Idaho	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Illinois	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Indiana	Y	Y	Y	Y	Y	Y	N	Y	Y/Y/Y	Y
Iowa	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Kansas	Y	Y	Y	Y	N	N	Y	Y	Y/Y/Y	Y
Kentucky	Y	Y	Y	Y	Y	Y	N	Y	Y/Y/N	Y
Louisiana	Y	N	Y	Y	Y	Y	Y	N	Y/Y/Y	Y
Maine	Y	Y	Y	Y	Y	Y	Y	Y	N/Y/Y	N
Maryland	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Massachusetts	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Michigan	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Minnesota	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/N	N
Mississippi	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/N	Y

Missouri	Y	Y	Y	Y	N	Y	Y	Y	Y/Y/N	Y
Montana	Y	Y	Y	Y	Y	N	Y	Y	Y/Y/Y	Y
Nebraska	Y	Y	Y	Y	Y	N	Y	Y	N/Y/Y	Y
Nevada	N	Y	Y	Y	N	N	Y	Y	Y/Y/Y	Y
New Hampshire	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
New Jersey	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
New Mexico	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
New York	Y	Y	Y	Y	Y	Y	Y	Y	N/N/N	Y
North Carolina	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/N	Y
North Dakota	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Ohio	Y	Y	Y	Y	Y	Y	Y	Y	N/N/Y	Y
Oklahoma	Y	Y	Y	Y	Y	Y	N	Y	N/Y/Y	Y
Oregon	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Pennsylvania	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/N	Y
Rhode Island	Y	Y	Y	Y	Y	Y	Y	Y	N/N/Y	Y
South Carolina	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
South Dakota	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Tennessee	Y	Y	Y	Y	Y	Y	Y	Y	N/N/N	Y
Texas	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Utah	N	Y	Y	Y	Y	Y	Y	Y	N/Y/Y	Y
Vermont	Y	Y	Y	Y	N	Y	Y	Y	N/Y/Y	Y
Virginia	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Washington	Y	Y	Y	Y	Y	Y	Y	Y	N/N/N	Y
West Virginia	N	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Wisconsin	Y	Y	Y	Y	Y	Y	Y	Y	Y/Y/Y	Y
Wyoming	Y	Y	Y	Y	Y	N	Y	Y	Y/Y/Y	Y

[a]Y, yes; N, no.
[b]All state public health laboratories have nucleic acid amplification test (NAAT) capability. The range of agents detected by NAAT varies.
[c]If no, rabies testing is performed by another state agency.

TABLE 2 State and territorial public health laboratories

ALABAMA
Bureau of Clinical Laboratories
State Department of Public Health
8140 AUM Dr.
P.O. Box 244018
Montgomery, AL 36124-4018
Phone: (334) 260-3400
Fax: (334) 274-9800

ALASKA
Department of Health & Social Services
Division of Public Health Laboratory
4500 Boniface Pkwy.
Anchorage, AK 99507
Phone: (907) 334-2100
Fax: (907) 334-2161
Website: http://www.hss.state.ak.us/dph/labs/

AMERICAN SAMOA
Department of Health Services
Government of American Samoa
LBJ Tropical Medical Center
Pago Pago, AS 96799
Phone: (684) 633-4606
Fax: (684) 633-5379

ARIZONA
Bureau of State Laboratory Services
AZ Department of Health
250 North 17th Ave.
Phoenix, AZ 85007
Phone: (602) 364-0741
Fax: (602) 542-0759
Website: http://www.azdhs.gov/lab/index.htm

ARKANSAS
Public Health Laboratory
Arkansas Department of Health & Human Services
P.O. Box 8182
Little Rock, AR 72203-8182
Phone: (501) 280-4079
Fax: (501) 661-2213
Website: http://www.healthyarkansas.com/pdf/testdirectory.pdf

CALIFORNIA
State Public Health Laboratory
California Department of Health Services
850 Marina Bay Pkwy.
Richmond, CA 94804
Phone: (510) 412-5846
Fax: (510) 412-5848
Website: http://www.dhs.ca.gov/ps/ls/

COLORADO
Laboratory Services Division
CO Department of Public Health & Environment
8100 Lowry Blvd.
Denver, CO 80230
Phone: (303) 692-3090
Fax: (303) 344-9989
Website: http://www.cdphe.state.co.us/lr/index.htm

CONNECTICUT
Division of Laboratories
CT Department of Public Health & Addiction Services
10 Clinton St.
Hartford, CT 06106
Phone: (860) 509-8500
Fax: (860) 509-8697
Website: http://www.dph.state.ct.us/Laboratory/state_laboratory.htm

DELAWARE
Public Health Laboratory
30 Sunnyside Rd.
Smyrna, DE 19977-1707
Phone: (302) 223-1520
Fax: (302) 653-2877
Website: http://www.dhss.delaware.gov/dhss/dph/lab/labs.html

DISTRICT OF COLUMBIA
Public Health Laboratory
Department of Health
300 Indiana Ave., NW
Suite 6154
Washington, DC 20001
Phone: (202) 727-8956
Fax: (202) 724-3927

FLORIDA
Bureau of Laboratories
Department of Health
Jacksonville Central Laboratory
1217 Pearl St.
Jacksonville, FL 32202
Phone: (904) 791-1500
Fax: (904) 791-1567
Website: http://www.doh.state.fl.us/lab/

GEORGIA
Public Health Laboratory
Department of Human Resources
1749 Clairmont Rd.
Decatur, GA 30033-4050
Phone: (404) 327-7900
Fax: (404) 327-7919
Website: http://www.health.state.ga.us/programs/lab/index.asp

GUAM
Department of Public Health & Social Services
P.O. Box 2816
Hagatna, GU 96932
Phone: (671) 735-7399
Fax: (671) 734-2066

HAWAII
State Laboratories Division
Hawaii Department of Health
2725 Waimano Home Rd.
Pearl City, HI 96782
Phone: (808) 453-6652
Fax: (808) 453-6662
Website: http://www.hawaii.gov/health/laboratories/index_html

IDAHO
Bureau of Laboratories
Department of Health & Welfare
2220 Old Penitentiary Rd.
Boise, ID 83712
Phone: (208) 334-2235
Fax: (208) 334-2382
Website: http://www.healthandwelfare.idaho.gov/site/3384/default.aspx

ILLINOIS
Department of Public Health Laboratory
825 North Rutledge St.
P.O. Box 19435
Springfield, IL 62702
Phone: (217) 782-6562
Fax: (217) 524-7924
Website: http://www.idph.state.il.us/about/laboratories/index.htm

INDIANA
Public Health Laboratory
State Department of Health
550 W. 16th St.
Indianapolis, IN 46202
Phone: (317) 233-8000
Fax: (317) 233-8003
Website: http://www.in.gov/isdh/22421.htm

IOWA
University of Iowa Hygienic Laboratory
102 Oakdale Campus, H101
Iowa City, IA 52242
Phone: (319) 335-4500
Fax: (319) 335-4555
Website: http://www.uhl.uiowa.edu/

(Continued on next page)

TABLE 2 State and territorial public health laboratories *(Continued)*

KANSAS
Division of Health & Environmental Laboratories
Department of Health & Environment
Forbes Building #740
Topeka, KS 66620
Phone: (785) 296-1535
Fax: (785) 296-1641
Website: http://www.kdheks.gov/labs/

KENTUCKY
Division of Laboratory Service
Department for Public Health
100 Sower Blvd., Suite 204
Frankfort, KY 40601
Phone: (502) 564-4446
Fax: (502) 564-7019
Website: http://www.chfs.ky.gov/dph/info/lab/

LOUISIANA
Louisiana Public Health Laboratory
DHH-OPH, Central Laboratory
3101 West Napoleon Ave., Suite 201
Metairie, LA 70001
Phone: (504) 219-4665
Fax: (504) 219-4452

MAINE
Health & Environmental Testing Laboratory
Department of Human Services
221 State St., Station #12
Augusta, ME 04333
Phone: (207) 287-2727
Fax: (207) 287-6832
Website: http://www.maine.gov/dhhs/etl/homepage.htm

MARYLAND
Laboratories Adminstration
Department of Health & Mental Hygiene
O'Conor Building
201 West Preston St.
Baltimore, MD 21201
Phone: (410) 767-6100
Fax: (410) 333-5403
Website: http://www.dhmh.state.md.us/labs/

MASSACHUSETTS
Bureau of Laboratories
State Laboratory Institute
305 South St.
Jamaica Plain, MA 02130
Phone: (617) 983-6200
Fax: (617) 983-6210
Website: http://www.mass.gov/dph/bls/labsite.htm

MICHIGAN
Public Health Laboratory
Department of Community Health
3350 North MLK Blvd.
Building 44
Lansing, MI 48909
Phone: (517) 335-8063
Fax: (517) 335-8051
Website: http://www.michigan.gov/mdch/1,1607,7-132-2945_5103---,00.html

MINNESOTA
Public Health Laboratory
601 Robert St. North
P.O. Box 64899
St Paul, MN 55164-0899
Phone: (651) 201-5200
Fax: (651) 201-5064
Website: http://www.health.state.mn.us/divs/phl/index.html

MISSISSIPPI
Public Health Laboratory
MS Department of Health
570 East Woodrow Wilson
Jackson, MS 39216
Phone: (601) 576-7582
Fax: (601) 576-7720
Website: http://www.msdh.state.ms.us/msdhsite/_static/14,0,188.html

MISSOURI
Public Health Laboratory
MO Department of Health
P.O. Box 570
101 N. Chestnut St.
Jefferson City, MO 65102
Phone: (573) 751-0633
Fax: (573) 751-7219
Website: http://www.dhss.mo.gov/Lab/

MONTANA
Laboratory Services Bureau
Department of Public Health
1400 Broadway
P.O. Box 6489
Helena, MT 59604
Phone: (406) 444-3444
Fax: (406) 444-1802
Website: http://www.dphhs.mt.gov/PHSD/Lab/Clinical/clinical-lab-index.shtml

NEBRASKA
Public Health Laboratory
University of NE Medical Center
981180 Nebraska Medical Center
Omaha, NE 68198-4080
Phone: (402) 559-2440
Fax: (402) 559-9497
Website: http://www.nphl.org/

NEVADA
State Laboratory–UNV
School of Medicine
1660 North Virginia St.
Reno, NV 89503-1738
Phone: (775) 688-1335
Fax: (775) 688-1460

NEW HAMPSHIRE
Public Health Laboratories
Division of Public Health Services
29 Hazen Dr.
Concord, NH 03301
Phone: (603) 271-4661
Fax: (603) 271-4783
Website: http://www.dhhs.state.nh.us/DHHS/PHL/default.htm

NEW JERSEY
Division of Public Health & Environmental Labs
NJ Department of Health & Senior Services
P.O. Box 361
John Fitch Plaza, 4th Floor
Trenton, NJ 08625-0361
Phone: (609) 633-2200
Fax: (609) 292-9285
Website: http://www.state.nj.us/health/phel/index.shtml

NEW MEXICO
Scientific Laboratory Division
New Mexico Department of Health
P.O. Box 4700
700 Camino de Salud NE
Albuquerque, NM 87196-4700
Phone: (505) 841-2500
Fax: (505) 841-2543
Website: http://www.sld.state.nm.us/index.asp

NEW YORK
Wadsworth Center
NY State Department of Health
P.O. Box 509
Albany, NY 12201
Phone: (518) 474-2160
Fax: (518) 474-3439
Website: http://www.wadsworth.org/

NORTH CAROLINA
State Laboratory of Public Health
Bath Building
306 N. Wilmington St.
Raleigh, NC 27601
Phone: (919) 733-7834
Fax: (919) 733-8695
Website: http://slph.state.nc.us/

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TABLE 2 State and territorial public health laboratories *(Continued)*

NORTH DAKOTA
Public Health Laboratory
Health Department
2635 East Main Ave.
P.O. Box 5520
Bismarck, ND 58502
Phone: (701) 328-6272
Fax: (701) 328-6280
Website: http://www.ndhealth.gov/microlab/

NORTHERN MARIANA ISLANDS
Department of Public Health
Commonwealth Health Center
P.O. Box 500409 CK
Saipan, MP 96950
Phone: (670) 234-8950

OHIO
Public Health Laboratory
State Department of Health
8995 East Main St.
Reynoldsburg, OH 43068
Phone: (614) 644-4590
Fax: (614) 752-9863

OKLAHOMA
Public Health Laboratory Services
OK State Department of Health
1000 NE 10th St.
Oklahoma City, OK 73117
Phone: (405) 271-5070
Fax: (405) 271-4850
Website: http://www.ok.gov/health/Disease,_Prevention,_Preparedness/Public_Health_Laboratory/

OREGON
State Public Health Laboratories
P.O. Box 275
Portland, OR 97207
Phone: (503) 693-4100
Fax: (503) 693-5602
Website: http://www.oregon.gov/DHS/ph/phl/

PENNSYLVANIA
Bureau of Laboratories
Pennsylvania Department of Health
110 Pickering Way
Lionville, PA 19353
Phone: (610) 280-3464
Fax: (610) 450-1932
Website: http://www.dsf.health.state.pa.us/health/cwp/view.asp?a=167&q=202401

PUERTO RICO
Public Health Laboratory
Department of Health
Commonwealth of Puerto Rico
Building A- Call Box 70184
San Juan, PR 00936-8184
Phone: (787) 274-6827
Website: http://www.salud.gov.pr/InstitutodeLaboratorios/Pages/default.aspx

RHODE ISLAND
Department of Health Laboratories
50 Orms St.
Providence, RI 02904-2283
Phone: (401) 222-5600
Fax: (401) 222-6985
Website: http://www.health.ri.gov/labs/index.php

SOUTH CAROLINA
Bureau of Laboratories
Department of Health & Environmental Control
8231 Parklane Rd.
Columbia, SC 29223
Phone: (803) 896-0800
Fax: (803) 896-0983
Website: http://www.scdhec.net/health/lab/index.htm

SOUTH DAKOTA
Public Health Laboratory
615 East Fourth St.
Pierre, SD 57501
Phone: (605) 773-3368
Fax: (605) 773-6129
Website: http://www.state.sd.us/doh/lab/index.htm

TENNESSEE
Laboratory Services
630 Hart Ln.
Nashville, TN 37247-0801
Phone: (615) 262-6300
Fax: (615) 262-6393
Website: http://health.state.tn.us/Lab/index.htm

TEXAS
Laboratory Services Section
Texas Department of State Health Services
1100 West 49th St.
Austin, TX 78756
Phone: (512) 458-7318
Fax: (512) 458-7294
Website: http://www.dshs.state.tx.us/lab/default.shtm

UTAH
Division of Epidemiology & Laboratory Services
46 North Mario Capecchi Dr.
Salt Lake City, UT 84113
Phone: (801) 584-8400
Fax: (801) 584-8586
Website: http://hlunix.hl.state.ut.us/lab/

VERMONT
Department of Health Laboratory
195 Colchester Ave.
P.O. Box 1125
Burlington, VT 05402-1125
Phone: (802) 863-7335
Fax: (802) 863-7632
Website: http://www.healthvermont.gov/enviro/ph_lab/lab.aspx

VIRGIN ISLANDS
Public Health Laboratory
3500 Estate Diamond
Charles Harwood Complete
Christiansted, St Croix, VI 00820
Phone: (340) 776-8311

VIRGINIA
Division of Consolidated Laboratory Services
600 North 5th St.
Richmond, VA 23219
Phone: (804) 648-4480
Fax: (804) 371-7973
Website: http://www.dgs.state.va.us/DivisionofConsolidatedLaboratoryServices/tabid/453/Default.

WASHINGTON
Public Health Laboratories
Department of Health
1610 NE 150th St.
P.O. Box 550501
Shoreline, WA 98155-9701
Phone: (206) 418-5450
Fax: (206) 418-5445
Website: http://www.doh.wa.gov/EHSPHL/PHL

WEST VIRGINIA
Office of Laboratory Services
State of West Virginia
Department of Health & Human Resources
167 11th Ave.
South Charleston, WV 25303-1137
Phone: (304) 558-3530
Fax: (304) 558-2006
Website: http://www.wvdhhr.org/labservices/

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TABLE 2 State and territorial public health laboratories *(Continued)*

WISCONSIN
WI State Laboratory of Hygiene
William D. Stovall Building
465 Henry Mall
Madison, WI 53706
Phone: (608) 262-1293
Fax: (608) 262-3257
Website: http://www.slh.wisc.edu/

WYOMING
Public Health Laboratory
Department of Health
2300 Capitol Ave.
517 Hathaway Building
Cheyenne, WY 82002
Phone: (307) 777-7431
Fax: (307) 777-6422
Website: http://wdhfs.state.wy.us/phsd/lab/index.html

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