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New Developments in Diagnostic Virology

Edited by Peter A. Bachmann

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Preface

The contributions to this book derived from the Seventh Munich Symposium on Microbiology on June 3 and 4, 1981, which was organized by the WHO Centre for Collection and Evaluation of Data on Comparative Virology at the Institute of Medical Microbiology, Infectious and Epidemic Diseases, University of Munich, Federal Republic of Germany. One of our principal purposes was to establish a forum at which the comparative aspects of questions of current interest in the field of medical virology could be discussed. In addition to the presentation of recent findings in microbiology, our overall aim was to crystallize trends and indicate new directions for future research activities.

This book is a topical review of "New Horizons in Diagnostic Virology." Everyone interested in virology is aware of the tremendous progress made in viral diagnostic techniques during recent years and the growing importance of viral diagnosis in human and veterinary medicine. There is yet another step that diagnostic virology has to take: the introduction on a routine basis of methods of molecular biology into the viral diagnostic laboratory.

The application of monoclonal antibodies and techniques for the chemical and biological identification of proteins, carbohydrates, and enzymes are discussed, as is the introduction of techniques for the characterization of nucleic acids in viral diagnosis.

Other chapters deal with the role of immune complexes and external photoscanning using radiolabeled antibodies and their part in future viral diagnosis. In addition, critical reviews are included on the potential as well as the limitations of such widely applied techniques as fluoro- and enzyme immunoassays, electron microscopy, demonstration of IgM antibodies, the use of protein A in viral diagnosis, concepts of automated systems, and computer-assisted filing and retrieval methods applicable in the field of viral diagnosis.

It may very well be that not all the techniques and methods presented here will eventually become routinely used in the diagnostic laboratory, but there is a good chance that new developments in viral diagnosis will comprise many of the procedures described in this book.

The success of any scientific undertaking is dependent on the help and cooperation of many individuals and institutions. We gratefully acknowledge the generous support of the Federal Ministry of Youth, Family and Health, and the Karl Friedrich von Siemens-Foundation. Likewise, we are grateful for the privilege of having had access to the expertise and advice of Drs. Fred Brown, Fritz Deinhardt, Pekka Halonen, Martin Kaplan, Hilary Koprowski, Anton Mayr, and Stanley Plotkin, all of whom were actively engaged in the organization of the programme. Finally, we would like to express our gratitude to Eileen Baum, who assisted in the editing of this book.

München, June 1982 Peter A. Bachmann

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Sense and Nonsense in Viral Diagnosis - Past, Present, and Future

KEN MCINTOSH*

1 Laboratory Viral Diagnosis: The First Century

Although infectious diseases have been recognized since the beginning of history, and diagnoses have been made for thousands of years from clinical signs or autopsy examinations, the first use of the laboratory to assist in the recognition of viral diseases awaited the development of the necessary tools: the light microscope and immunologic systems which would detect minute amounts of antigens or antibodies. The first laboratory-based diagnosis of a viral disease can probably be attributed to a Scotsman, *James Brown Buist*, who in 1886 stained the lymph obtained from the skin lesions of patients with smallpox and saw with almost miraculous acuity "elementary bodies", which he took to be the cause of the disease (*Gordon* 1937). He called these the spores of micrococci and estimated their size to be 150 nm. We now know that he was seeing the aniline-stained poxvirus particles and underestimated their diameter by at least a factor of 2. This was 6 years before the discovery of viruses, for it was only in 1892 that *Ivanovski* described a transmissable, filterable agent as the cause of tobacco mosaic disease. *Buist* had not only pioneered in laboratory viral diagnosis but even in "rapid" viral diagnosis, since his technique could be completed in hours and he was using the simple but even today somewhat unorthodox approach of examining clinical specimens directly by microscopy.

Over the next few decades a number of communicable diseases, beginning with yellow fever, were shown to be caused by viruses, and animal inoculation was the sole laboratory technique used for laboratory diagnosis. The first use of embryonated eggs for the growth of viruses was, curiously enough, the demonstration by *Rous* and *Murphy* in 1911 that they could grow tumors by injecting cells (and later filtered homogenates) into chick embryos through a window cut in the shell. This technique was only slowly adapted to the growth of other viruses, beginning in 1931 with fowlpox by *Woodruff* and *Goodpasture.*

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Meanwhile the science of tissue culture was slowly evolving. However, the usefulness of tissue culture for diagnostic purposes had to await the discovery that viruses could not only grow in such in vitro cultures but could destroy cultures as well, and thus be recognized. Although vaccinia was probably grown in explants of rabbit cornea in 1913 (*Steinhardt* et al. 1913), the first visible cytopathic effect was recorded for the lymphogranuloma venereal agent in 1939 by *Gey* and *Bang* and then for vaccinia in 1940 by *Feller* et al.

It seems ironic that just as the very first laboratory viral diagnosis had been by direct detection of the virus particles in smallpox lesions by *Buist* in 1886, smallpox antigen was detected in clinical materials as early as 1932, before it could be shown to produce a visible cytopathic effect in tissue culture. *Parker* and *Muckenfuss* (1932) developed the complement fixation technique for direct detection of smallpox antigen in lesion fluid at about the same time the virus was first grown in embryonated eggs; this antigen detection test became the procedure of choice for laboratory diagnosis of smallpox for the next several decades.

With the great advances in tissue culture technology, including the development of roller drums (*Gey* 1933) and diploid cell strains (*Hayflick* and *Moorehead* 1961), direct microscopy and antigen detection fell into disuse as diagnostic tools. It may be that the excellence of the new tissue culture technology paradoxically held up advances in diagnostic techniques by lulling virologists into a sense of complacency that this was the best that could be done with most of the known viruses. One simply had to wait the days or weeks that most viruses required to produce characteristic cytopathic changes, and accept the fact that the patient would be discharged or dead before the virus was identified.

Consistent with this analysis is the fact that both antigen detection and direct electron microscopy of the clinical specimen were developed primarily as tools to detect viruses which would not grow at all in tissue culture: hepatitis B (and A) and the diarrhea viruses. "Necessity is the mother of invention" is the usual homily applied to this sort of situation, but a better one might be "Serendipity is the fairy godmother of progress."

Now we are in a period when we have both tissue culture technology and the newer (and older) tools of direct microscopy and antigen detection. Viral diagnostic laboratories are no longer the sole and undisputed possession of state health departments, nor are academic laboratories the only loci of virologic research. Smaller private and public hospitals, profit-oriented independent laboratories, and larger reagent manufacturers are all in the business of serving the consumers' needs for viral diagnosis and are producing an array of diagnostic tests which is as bewildering for the physician as it is dazzling for the patient. It seems inevitable that there should be nonsense as well as sense in this process.

2 Laboratory Viral Diagnosis: Sense and Nonsense

The use of the viral diagnostic laboratory makes sense when it does at least one of two things: when it teaches the physician or the patient something which renders him a better physician or patient, and when it leads to action toward better health.

The teaching role of the laboratory is often overlooked. There is enormous ignorance about viral diseases both in the professional and lay public. When the doctor tells his patient, "You have a virus, or whatever it is that is going around," he is thinly concealing his cavernous ignorance of a group of diseases which are often of great concern to patients – ignorance of their etiology, epidemiology, prognosis, communicability, and even their complications, drug interactions, and public health effects. All of this ignorance is accepted by the medical profession on the grounds that nothing can be done anyway. However, ignorance is not bliss and it may even be disastrous, as for example when a patient has a heart attack during the influenza season and is passed off wearily and without thought as another case of the flu.

When a physician sets out to find out what virus is involved in a particular clinical syndrome, and when the diagnostic laboratory answers his search with a prompt and specific reply, that physician has been taught something about the syndrome that he is unlikely to forget. If this combination of virus-disease information is properly processed and recorded in his mind – the clinical presentation, season of the year, epidemiologic setting, cause-and-effect probability, diagnostic procedure, prognosis – he is a better physician at the end of it. Moreover, he may not necessarily have to use the diagnostic laboratory the next time to continue to profit from the information gained.

The patient, too, is the object of the teaching process, probably as often as the physician. The parents of a child who is hospitalized with pneumonia regard the event as a crisis in their lives. The problem may be routine to the physician, but it certainly is not to the family. They often want to know exactly what it is that is causing the illness, where it was acquired, who else can get it, when it will be over, whether it does permanent damage, and innumerable other facts which can only be obtained (if at all) when the etiology of the illness is known. Thus, when the specific viral diagnosis teaches, it makes sense, and although it is difficult to place a monetary value on this process, one can often say that the cost of such information is "worth it." This becomes particularly true as the prices of the tests fall.

The more commonly accepted role for diagnostic virology is as a trigger to action of some kind. Few would argue that the timely discovery of herpes simplex in the female genital tract at term, which leads to cesarean section and thus a lower risk to the baby, is sensible use of the diagnostic virology laboratory. Similar arguments can be made for the laboratory confirmation of a clinical impression before the use of antiviral drugs such as adenine arabinoside or acycloguanosine; or detection of rabies immunofluorescence in the brain of a skunk which has just bitten a child. In a sense, the intelligent use of viral diagnostic methods for public health purposes also leads directly to action. This is the traditional forum for viral diagnosis – keeping watch over epidemic diseases such as influenza, equine encephalitis, poliomyelitis, rabies, or measles, often to guide immunization programs, sometimes to warn communities, and to assist in planning.

It is often said that one of the values of rapid and precise viral diagnosis is to guide the withdrawal of antibiotics. This is not as simple as it sounds, and caution is necessary to separate sense from nonsense in this area. Undoubtedly, there are certain, often complex, situations where the identification of a virus simplifies the therapeutic choices to be made, but in my experience these are uncommon. As viral diagnosis improves, particularly for the viruses causing meningitis and encephalitis,

this will become a more frequent appropriate use for the diagnostic laboratory. However, more often than not, this particular issue has a component of nonsense to it. Why should that be? I shall illustrate this point by what I call the viral pneumonia paradox.

In young infants and children between 3 months and 3 years of age, virus-induced pneumonia is universally conceded to be much more common than bacteria-, mycoplasma-, or chlamydia-induced pneumonia. Despite this awareness, however, virtually every child whose chest X-ray shows the acute infiltrates of pneumonia is placed on antibiotics. Moreover, even though the laboratory might return a diagnosis of, say, parainfluenza virus type 3 within 24 h of receiving an admission respiratory specimen, the child is nevertheless continued on ampicillin in the hospital and sent home on amoxicillin. "I can't take the chance," is one rationale. "Can you assure me that there are not some pneumococci down there aggravating the problem?" is a question I am often asked. Parents perpetuate the problem when they say (often at a dinner party), "My pediatrician finally took an X-ray and it showed pneumonia, so now, thank God, we have him on antibiotics." The child's subsequent recovery is taken as evidence of bacterial involvement in the process.

In my experience, diagnostic information about the virus in the respiratory secretions if often sought but ignored. The physician follows his better instincts in culturing, but when he is confronted with a viral diagnosis he baulks at withdrawal of antibiotics. There are two reasons for this. First, we really do know very little about the possible role of bacteria in viral pneumonias. Just as important, however, is our reluctance to stop all drug treatment of a sick patient in response to diagnostic information. The first problem will be solved only when we develop more definitive diagnostic procedures which describe precisely the relationship between the agent and the host (rather than just what the agent is). The second will dissolve when we have safe and effective antivirals. Thus, the viral diagnosis of pneumonia, and sometimes also of gastroenteritis, is nonsense when the information is requested and then discarded as irrelevant. A related misuse of the laboratory is the unthinking measurement of antibody to the "TORCH" group in any child of any age who is not behaving right. In most instances, little consideration is given to the meaning of the information which will result from such a screening test, and the resultant data are useless.

Sending the wrong specimen is a very common sort of nonsense we have to deal with. A common misconception is that the best way to find the viral cause of aseptic meningitis or some other acute neurologic problem is to send an isolated cerebrospinal fluid sample for cultures. In fact, the physician who does this might strike gold and recover a virus in the most incriminating body fluid, but far more likely he will find none and remain ignorant. It is better to culture stool and respiratory tract as well as cerebrospinal fluid, and thereby obtain a suggestive, albeit not definitive, isolate. We have two perhaps oversimplistic rules which help to overcome this problem: first, if an illness is on a surface (skin mucous membranes), culture that surface or its secretions and nothing else; second, if an illness is hidden or systemic, culture multiple sites.

Still another bit of nonsense is the culture taken as an afterthought as the patient, now recovered, is getting ready to go home. One of the most difficult misconceptions to set straight is the idea that all materials for viral culture should be immediately placed in the freezer if there is a likely delay of more than a few minutes. We now preach that specimens for viral culture should never be frozen, and we have improved our

isolation rates. There are infinite ways that a laboratory can be misused. Running a successful clinical diagnostic service is an unending educational task.

3 The Future

The immediate prospect for rapid, convenient, and inexpensive identification of many viruses from clinical specimens seems quite bright and close. Indeed, in some places in the world where laboratories are well developed and have put particular emphasis on antigen detection or electron microscopy, it is happening now. Nevertheless, viral diagnosis has a long way to go before it reaches maturity.

To serve our public better, first, we need tests which are still cheaper, faster, and simpler. It is curious that one of the best examples of such a test is the Monospot test, or its equivalent. The test is so simple and inexpensive that it is done in all hospitals and many office laboratories. The fact that we have no understanding of the mechanism by which the test works in no way detracts from its usefulness. It is a worthy goal toward which we can aim.

Second, we need some tests that are less specific. An inexpensive test to distinguish viral from nonviral disease, without regard to which virus, would be enormously valuable in many minor acute illnesses. Many attempts have been made of course: *C*-reactive protein, sedimentation rate, the nitroblue tetrazolium test, and white blood cell and differential count have all been investigated and used in the attempt to differentiate between bacterial and viral disease. The specificity and sensitivity of these tests are the subject of innumerable published reports. They are, however, despite their widespread use, inadequate for the task. It may be that some rapid assay for interferon would be more virus-specific than any of the existing tests. Perhaps there are other side products of virus-infected cells, neglected until now, which could be found in body fluids. On the other hand, the best approach might be from the other end; are there products common to bacteria such as peptidoglycan or muramic acid that can be found? This would be viral diagnosis by exclusion.

Third, we need better methods of diagnosing the presence of viruses plus tissue damage. This applies particularly to the respiratory viruses, where the presence of virus does not guarantee its importance in disease; but the same principles might also apply to enteroviruses, cytomegalovirus, and others as well. It seems possible that radionucleotide scans could localize the involvement of a virus in a particular tissue as they do bacteria in, say, bone. Perhaps labeled antibody could play such a function in vivo as it does in solid-phase immunoassays in vitro. Another task would be to search for specific viral products produced on infection of certain tissues. For clinicians such tests would be of enormous value, not only in virology but in all categories of infections disease.

Finally, when working with certain viruses it would be efficient to recognize certain inherent limitations in the tools we have and try other tools in an effort to circumvent the problem. Some viruses are present in low titer and will not be detectable by existing antigen detection systems. Others are small, and electron microscopy searches are long and frustrating. Others do not circulate, and looking in the blood is fruitless. In other cases, the serum antibody response is weak or nonexistent, and it makes little sense to use serologic methods for diagnosis. Rather than persisting along lines

where the tools are too blunt or too weak, we should be watching out for new approaches.

In conclusion, we should look forward to a time when the consumers of the system understand the tests they are using and order them when they need them and for appropriate indications. This will require both a simplification of the systems and also education of the physicians and the lay public. It is part of our responsibility in emphasizing the sense and eliminating the nonsense in viral diagnosis.

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Immune Complexes in Viral Infection

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1 Introduction

The development of disease during viral infection may result from at least two major mechanisms, which are often combined. First, viruses can cause cell damage, directly or through the release of toxic products. Second, the cellular and humoral branches of the immune response directed against antigen(s) related to the infectious agent can also result in tissue injury. Although the production of antibody and its subsequent binding to antigen during the course of an immune response lead, in most cases, to

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antigen elimination, in vivo formation of immune complexes represents a major pathway for the pathological expression of the immune response. Indeed, immune complexes are formed when antibodies are produced and react with antigen molecule(s) persisting in the host or which are released from cells into extracellular fluids. In addition, some viruses are known to induce a polyclonal B cell proliferation resulting in the production of antibodies with a broad spectrum of specificity, including autoantibodies, and possibly lead to the formation of immune complexes involving self antigens or modified self antigens.

1.1 The Nature of Immune Complexes

The formation, structure, and properties of immune complexes depend on the nature of the antigen, of the antibody, of their interaction, and on the secondary binding of immune complex-reactive molecules. The size and charge of the antigen and its possible reactivity with various biological structures, influence the size and the biological activities of immune complexes. This is particularly relevant for viruses or viral glycoproteins that can bind per se to cell membranes and/or to free molecules. Viruses which display some affinity for C1q (*Cooper* et al. 1976) can react more efficiently with C1q when presented in an aggregated form. Viral glycoproteins expressed at the cell surface, e.g., those in measles virus – infected cells, can bind C3, leading to the triggering of the alternative complement pathway, but without activation of the term-

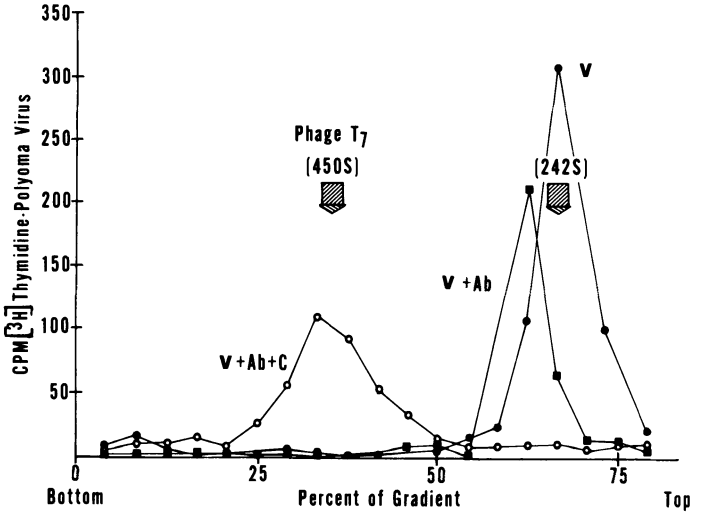

Fig. 1. [^{3}H]thymidine-labeled polyoma virus (*V*) was incubated with antibody (*Ab*) and complement (*C*) and then placed on a 5%–20% sucrose density gradient for rate-zonal centrifugation analysis. The sizes of the complexes were 270S and 450S for *V + Ab* (■——■) and *V + Ab + C* (○——○), respectively. [^{3}H]thymidine-labeled DNA was not susceptible to deoxyribonuclease digestion. Note the enhanced aggregation of antigen and antibody molecules following addition of complement. There is no evidence for lysis of the virus. Further electron-microscopic study of the *V + Ab + C* complex showed marked enhancement of clumping and covering of polyoma virus particles over that seen with *V + Ab* alone. No pits or craters were seen on the virus membrane. Polyoma virus (*V*, ●——●) and Phage T_7 were used as mobility markers (*arrows*, 242S and 450S, respectively)

inal (C5–C9) sequence of attack to the membrane. Subsequent binding of specific antibody to the glycoproteins results in activation of C5–C9 and lysis of the cell (*Sissons* and *Oldstone* 1979). Polymeric antigens which carry a single determinant in repetitive representation, e.g., glycoproteins present on the surface of virions or infected cells, can induce the formation of immune complexes involving antibodies with a restricted specificity. Alternatively, immune complexes involving antibodies with multiple specificities can be formed when antigens carrying several different determinants are involved. Evidently, a molecule with a single antigenic determinant can only form complexes containing a single antibody molecule (*Steensgaard* et al. 1977).

The antibody involved in immune complex can be one of the various immunoglobulin (Iq) classes and subclasses, and thus have different types of biological activity. The *affinity* of the antibody for the antigen in the complex will also critically influence the biological activity of the immune complex (*Eisen* 1980). The *ratio* of antigen to antibody molecules and their relative *concentrations* determine the size, solubility, and biological reactivity of the complex. Finally, according to the *nature of the complex*, different types of molecules can be secondarily bound: complement components, such as C1q, C1r, C1s, C4, and C3; antibodies with anti-Iq activity, either anti-Fc activity (7S antiglobulins or rheumatoid factor) or anti-$F(ab')_2$ activity; anti-idiotype antibodies; or antibodies against fixed C3 and C4 (immunoconglutinins). The binding of these molecules to a small-size complex results in the formation of a larger complex (Fig. 1) with new physical and biological properties.

1.2 The Site of Formation and the Fate of Immune Complexes

In vivo, the fate and the effects of immune complexes are directly dependent on the site of their formation as well as on the nature and relative concentrations of the antigen and antibody. In extracellular fluids, immune complexes can be formed subsequent to the binding of antibody to a cell-free antigen or to an antigen present on the cell membrane. The binding of antibody to cell-free viral antigens results in the formation of cell-free immune complexes, and is likely to be a major mechanism of formation of immune complexes during viral infections involving nonbudding viruses. Antibody-binding material can consist of intact virion particles or viral polypeptides, or DNA or RNA derived from microorganism destruction.

In general, the reaction of antibody with antigen present on the cell surface results in the formation of cell-bound immune complexes. The cell surface-associated antigens can be passively attached to the membrane, as with whole virions, viral polypeptides or DNA fragments with cytophilic activity. Similarly, double- and single-stranded DNAs have a high affinity for kidney glomerular basement membrane and can function as planted antigens leading to the in situ formation of immune complexes (*Izui* et al. 1976) Surface-associated viral antigens can form a new surface structure after infection of the cells by enveloped viruses. Antibodies to free virions can also bind to viral glycoproteins expressed at the surfaces of infected cells. Moreover, it has been demonstrated recently by clonal analysis that antibodies specific for viral-modified and/or viral-associated major histocompatibility complex (MHC) structures can be produced after influenza infection (*Wylie* et al. 1982). The formation of cell-bound

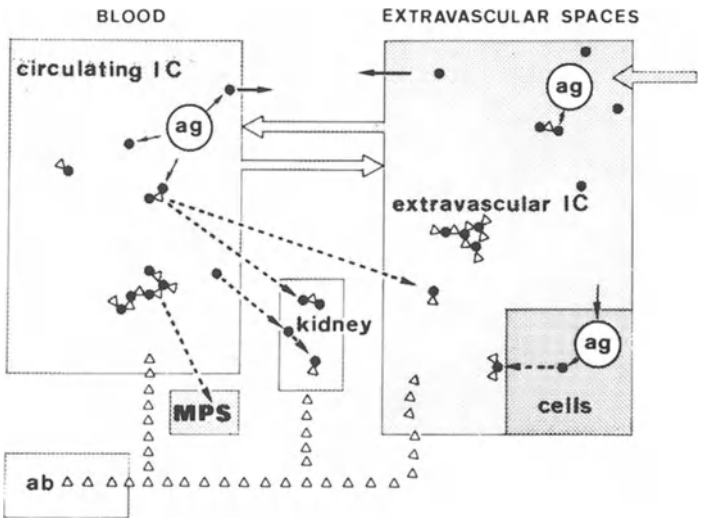

Fig. 2. Possible sites of formation of immune complexes (*IC*) in vivo may be in the blood or in extravascular spaces, according to the localization of the antigens at the time when antibodies appear (*ag* represents a source of antigen, e.g., a virus). The fate of the complexes is dependent on the site of their formation. *MPS* represents the mononuclear phagocyte system, mainly the liver and the spleen. (*Casali* et al. 1979)

immune complexes can result in subsequent release of the complexes. For example, antibodies can bind to new antigenic determinants expressed on the surface of cells infected by measles virus. After polar redistribution (capping) of these determinants and of the bound antibodies, the surface-bound immune complexes can be released into the extracellular medium.

In vivo, extracellular immune complexes involving cell-free or cell-bound antigens can form in the circulation or in extravascular fluids, or in both compartments (Fig. 2). The fate of immune complexes in circulating blood depends mainly on their size. Large-size complexes, either complement fixing or non-complement-fixing, are almost completely cleared from the circulation within a few minutes by the mononuclear phagocyte system (*Mannik* et al. 1974; *Cochrane* and *Koffler* 1973; *Haakenstad* and *Mannik* 1977). Such complexes are actively concentrated in the liver by Kupffer cells. Small-size, and particularly non-complement-fixing immune complexes are poorly cleared from the circulation and, in some instances, can be fixed in vessel walls or in filtering membranes such as renal glomeruli or choroid plexus. Immune complexes have been found in renal glomeruli following a variety of viral infections such as lymphocytic choriomeningitis virus (LCMV) and lactic dehydrogenase virus (LDV) infection in mice (*Oldstone* and *Dixon* 1970a, b; 1971a, b). The presence of immune complexes at the level of choroid plexus has also been reported (*Oldstone* 1975).

Immune complexes formed in extravascular spaces are not cleared as rapidly as circulating immune complexes and may induce inflammatory foci. The pathological consequences will depend on the concentration and persistence of the complexes. In some clinical conditions an exchange between the extravascular and the intravascular pool of immune complexes may be observed, but this is not a general rule

and typical immune-complex disease may occur in the absence of circulating immune complexes.

1.3 Biological Activities of Immune Complexes

The biological properties of immune complexes are largely related to the presentation of antigen and/or antibody molecules in an aggregate form, and therefore at a higher density compared with that of the corresponding free molecules. Aggregated antigens or antibodies are bound more avidly by cellular or humoral receptors than are their isolated counterparts. The biological effects of immune complexes are first determined by their interaction with these receptors (recognition step) (*WHO* 1977; *Zubler* and *Lambert* 1977; *Casali* et al. 1979; *Theofilopoulos* and *Dixon* 1979). The binding of immune complexes to the classical humoral receptor, the first component of complement, and particularly its C1q subunit, can trigger the activation of the complement system and its effector mechanisms (*Muller-Eberhard* 1975). At cell surfaces, the binding of immune complexes may first occur specifically to cell receptors for antigens, possibly initiating cellular events involved in the immune response (*Warner* 1974; *Pepys* 1976); moreover, an antigen-nonspecific binding occurs on many cells which have receptors for the Fc part of Ig molecules (*Spiegelberg* 1974) or for complex-bound complement components (*Nussenzweig* 1974). The biological activities of immune complexes include activation of plasma components, mainly the complement system, and activation of cells.

1.3.1 Activation of Complement

Immune complexes can activate the complement system through both the classical and, in some cases, the alternative pathways (*Muller-Eberhard* 1975; *Muller-Eberhard* and *Schreiber* 1980). As a result of activation of the complement system, several biological activities are generated that play a role in diseases of immune-complex origin. These include:

1. *Solubilization of the antigen-antibody complexes.* Insoluble antigen-antibody complexes prepared in vitro become soluble after incubation in fresh serum at 37 °C (Fig. 3). The solubilization occurs, although to a limited extent, in C2- or C4-deficient serum but does not occur if the alternative complement pathway is defective (*Czop* and *Nussenzweig* 1976). This phenomenon does not seem to involve an enzymatic attack on the immune complex; after their solubilization, the antigen and the antibody can be recovered with no change in their molecular weights. Presumably, the recruitment of some complement factors, mainly C3 and properdin factor B, into the preformed lattice allows for deaggregation of the antibody molecules stuck together through their Fc portion, and partial dissociation of the antigen from the antibody. The solubilization of antigen-antibody complexes in the presence of complement could have important in vivo implications in viral diseases. A reduced clearance of immune complexes from the circulation and a greater tendency for their deposition in tissues would be expected in acquired or primary complement deficiency.

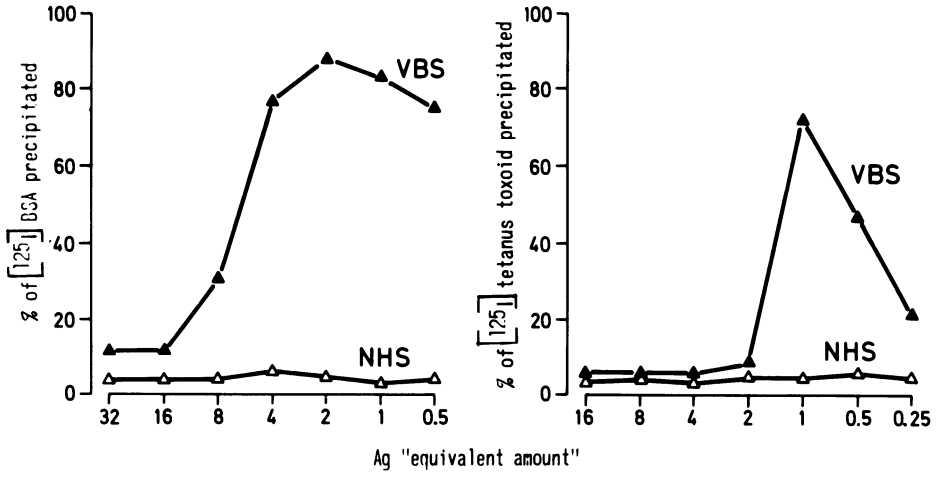

Fig. 3. Solubility of [^{125}I]BSA–anti-BSA and [^{125}I]tetanus toxoid–anti-tetanus toxoid complexes after 1 h at 37 °C and 72 h at 4 °C. Immune complexes were prepared either in veronal buffered saline (*VBS*) or in fresh normal human serum (*NHS*), with constant amounts of antibody (anti-BSA, 60 μg/ml; anti-tetanus toxoid, 80 μg/ml) and decreasing concentrations of antigen (*Ag*). Following incubation and centrifugation at 1500g for 15 min, the radioactivity of the unwashed precipitate was measured and its value expressed as a percentage of the total radioactivity added to the sample. (*Casali* et al. 1979)

2. *C3 immune adherence.* This is a phenomenon in which leukocytes bind to C3b on membranes or to immune aggregates at the point of complement fixation (*Nussenzweig* 1974; *Ross* and *Polley* 1975; *Theofilopoulos* et al. 1977). Membrane-bound C4b also induces the immune adherence of certain cells, although to a lesser extent than C3b. The immune adherence capacity of C3b is rapidly destroyed by C3b inactivator.

3. *Chemotaxis.* Chemotaxis of leukocytes has been shown to be a property of activated complement components. The C5-6-7 complex and C5a are known to possess this capacity (*Muller-Eberhard* 1975; *Taylor* et al. 1977; *Fernandez* et al. 1978).

4. *Exocytosis of neutrophil granules.* Immune adherence through the C3b receptor leads to exocytosis of neutrophil granules when phagocytosis cannot occur.

5. *Anaphylatoxin activity.* C3a and C5a can also stimulate mast cells to release their granules (*Hugli* and *Muller-Eberhard* 1978).

6. *Lysis of cells.* Cells are lysed by the action of the terminal complement components when an immune complex is formed between an antigen present on a cell and a complement-activating antibody, or when an immune complex is brought into close apposition with the surfaces of certain cells. Whereas the first condition leads to direct lysis, the second is a reaction of indirect lysis, with the terminal components of complement (C5–C9) binding to the bystander cell (*Thompson* and *Lachmann* 1970).

1.3.2 Interactions with Cells

Immune complexes can activate a variety of cells by interacting with various surface receptors. Cellular activation can have several biological consequences. These reactions are discussed in this section.

1. *Platelets.* Human platelets bear receptors for the Fc portion of Ig, and they clump and release nucleotides and vasoactive amines subsequent to binding of immune complexes or aggregated Ig. All subclasses of IgG produce this response in platelets (*Henson* and *Spiegelberg* 1973). Platelets also respond to thrombin, adenosine diphosphate, collagen, prostaglandin, platelet-activating factor of basophils and mast cells, adrenalin, and many other agents, by clumping and releasing vasoactive amines and the phospholipid procoagulant, platelet factor 3 (PF3). The data available strongly suggest a primary role for platelets in the expression of immunopathological lesions of immune-complex diseases (*Kekomaki* et al. 1977). For instance, they likely play a key role in massive intravascular coagulation occurring during dengue virus infection.

2. *Neutrophils.* Human neutrophils have receptors for the Fc region of IgG (*Spiegelberg* 1974) and for fragments of C3, C4, and C5 (*Anwar* and *Kay* 1977). Attachment of particles coated with either IgG1 or IgG3 antibody is followed by phagocytosis or contact-dependent lysis. Contact between neutrophils and aggregated Ig or immune complexes leads to the release of granules from the cells, with liberation of proteolytic enzymes and basic peptides. These substances increase vascular permeability, stimulate mast cells, and generate thromboplastin, which activates the intrinsic clotting system. In human systems, IgG1, IgG2, IgG3, IgG4, and IgA in aggregated form, and C3b, C4b, C3bi, C3a, and C5a have been reported capable of such stimulation. IgG Fc and complement receptors contribute to the process of phagocytosis of immune complexes by neutrophils. Complement receptors seem to be involved primarily in the process of attachment of immune complexes to the cell surface, whereas engagement of IgG Fc receptor is necessary for the triggering of phagocytosis (*Schribner* and *Fahrney* 1976). The process of exocytosis of neutrophil granules is probably responsible for much of the injury in acute immunologic nephritis (there is also an important neutrophil-independent mechanism), acute immunologic synovitis, arthritis, and the vasculitis of the Arthus reaction. The release of superoxides, singlet oxygen, and peroxide may also play a role in the injurious process.

3. *Basophils and mast cells.* Basophils have the capacity to bind IgE antibody and to a lesser extent some cytophilic IgG antibodies (*Becker* and *Henson* 1973; *Spiegelberg* 1974; *Ishizaka* 1975). Following the interaction of this fixed antibody with multivalent antigen, the contents of the basophil granules are exocytosed. The granules contain heparin, histamine, slow-reacting substance of anaphylaxis (SRS-A), the eosinophil chemotactic factor of anaphylaxis (ECFA), and the platelet-activating factor (PAF). Similar exocytosis can also result from the reaction of the basophil with the complement fragments C3a and C5a (*Hugli* and *Muller-Eberhard* 1978; *Glovsky* et al. 1979). The receptors for these two closely related fragments are distinct. Basophils and mast cells have assumed significance in the deposition of circulating immune complexes in acute experimental serum sickness of rabbits. An IgE-mediated

anaphylactic triggering accompanies the deposition of circulating complexes and appears to be responsible for the increased vascular permeability which may be essential for the deposition of the circulating complexes in arteries and glomeruli (*Cochrane* and *Koffler* 1973; *Cochrane* and *Dixon* 1978; *Wilson* and *Dixon* 1976; *Wilson* 1981).

4. *Mononuclear phagocytes.* Human mononuclear phagocytes ("monocytes" in circulation and "macrophages" in tissue spaces) bind monomeric IgG1 and IgG3 by the Fc portion (*Spiegelberg* 1974). Once aggregated, IgG antibodies of any subclass bind to mononuclear phagocytes and induce secretion of hydrolytic enzymes but not cell death (*Holm* and *Hammerstrom* 1973; *Cardella* et al. 1974). Mononuclear phagocytes also have receptors for C3b ad C3d. IgG Fc receptors mediate binding and ingestion of particulate complexes, whereas C3b and C3d receptors mediate binding only of C3-coated, sensitized particles (*Bianco* et al. 1975; *Ehlenberger* and *Nussenzweig* 1977). However, C3b and C3d receptors have a synergistic effect on phagocytosis of opsonized particles bearing fixed C3 molecules (*Ehlenberger* and *Nussenzweig* 1977). Immune complexes phagocytosed by macrophages are degraded by lysosomal enzymes (*Cruchaud* et al. 1975). Finally, following phagocytosis, the macrophages release hydrolytic enzymes that participate in inflammatory processes.

5. *Lymphocytes.* Most, if not all, human B cells carry receptors for IgG Fc, C3b–C4b, C3d, and C1q (*Nussenzweig* 1974; *Theofilopoulos* et al. 1974, 1977; *Dickler* 1976; *Sobel* and *Bokisch* 1975; *Tenner* and *Cooper* 1979). These receptors are discrete entities, and immune complexes with fixed complement appear to bind to lymphocytes almost exclusively through complement receptors (*Theofilopoulos* et al. 1974; *Kammer* and *Schur* 1978). The majority of human T cells express receptors for the Fc portion of either IgG or IgM (*Moretta* et al. 1977, 1978, 1979). A fraction of antigen-stimulated T cells carries receptors for C3b but not C3d. Human killer cells, capable of antibody-dependent, cell-mediated cytolysis, are characterized by relatively high affinity Fc receptors (*Perlmann* et al. 1977, *Spiegelberg* et al. 1976), but they appear to be heterogeneous as far as complement receptors are concerned. Natural killer (NK) cells possess high-affinity IgG Fc receptors but not complement receptors (*Perussia* et al. 1979).

Immune complexes can modulate the humoral and cellular branches of the immune response both in vivo and in vitro by interacting with B and T cells bearing Fc, complement, and/or antigen receptors on cellular membranes. Immune-complex modulation of the immune response is largely dependent on the molar ratio of antigen to antibody, the epitope diversity of the complex, the steric conformation of the antigen, and the nature of the antibody. In addition to immune complexes, anti-idiotypic antibodies certainly play an important role in the modulation of the immune response (*Eichmann* and *Rajewsky* 1975; *Eichmann* 1975). Immune complexes have been found both to enhance and to suppress lymphocyte activation induced experimentally by immunologically specific and nonspecific stimuli (*Dukor* et al. 1974; *Pepys* 1974, 1976; *Dierich* and *Landen* 1977). Some of these differences may be explained by the particular composition and configuration of the complexes, which may determine whether it reacts with lymphocytes through the antigenic determinants or through the Fc portion of the antibody. The activity of Fc receptor-bearing T and B

lymphocytes can be modulated via this receptor. The effects of immune complexes on B cell responses would likely depend on the stage of differentiation of the B cells. There is considerable evidence that mature B cells can undergo inactivation following brief in vitro exposure to immune complexes (*Feldman* and *Nossal* 1972). Plasma cells are also susceptible to alterations in function by binding multivalent antigens, anti-idiotypic antibodies (*Eichmann* 1978), or immune complexes (*Nossal* and *Schrader* 1975). Immune complexes also appear to account for inhibition or activation of T cells in several situations. Immune complexes involving IgM or IgG antibodies could modulate the helper and suppressor function in antibody production through their binding to T cells bearing either IgM or IgG Fc receptors, respectively (*Moretta* et al. 1977, 1979).

At the effector cell level, antigen-antibody complexes in antigen excess can efficiently inhibit killer cell functions via saturation of their Fc receptors (Fig. 4). Relevant antigen-antibody complexes in antibody excess can, however, enhance killer activity by "arming" the killer cells (*Saksela* et al. 1975; *Lustig* and *Bianco* 1976) (Fig. 4). NK cell activity can also be abrogated by immune complexes, although reports in this regard are contradictory (*Kay* 1980). Antigen-antibody complexes may even inhibit T cell-mediated cytolysis by blocking cytotoxic T lymphocyte (CTL) receptors. At least some of the serum blocking factors found in the serum of animals and humans with viral infections or tumors consist of antigen-antibody complexes. In the case of viral infections, it is likely that antibodies specific for the MHC-viral polypeptide complex (*Wylie* et al. 1982) can bind enveloped virions bearing MHC determinants in the cell-free phase. In antigen excess, these virion-antibody complexes could bind CTLs directed against the infected cells from which the free virions originated (Fig. 4).

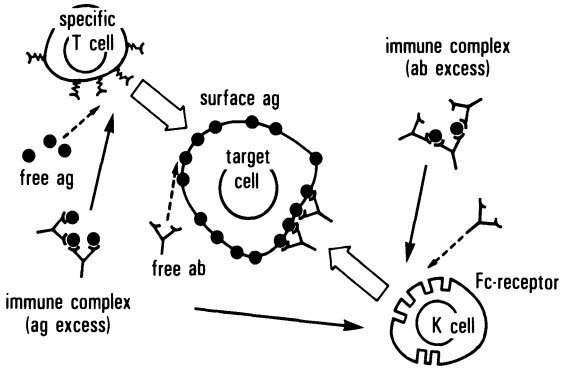

Fig. 4. Interference of immune complexes at the cytotoxic cell level. *Right side of the panel:* immune complexes (involving relevant or irrelevant antigen molecules), in antigen (*ag*) excess, can inhibit the function of killer (*K*) cells by saturation and subsequent modulation of their surface Fc receptors. In antibody (*ab*) excess, immune complexes involving relevant antigen molecules can enhance killing of appropriate target cells by K cells. *Left side of the panel:* antibody specific for major histocompatibility complex (MHC)-associated and/or modified viral products (see Sect. 1.2) could bind cell-free enveloped virions presenting identical determinants and form immune complexes. In *ag* excess, these immune complexes could bind a specific cytotoxic T cell directed against the infected cell from which the virions had originated

1.4 Tissue Injury Induced by Immune Complexes

The formation of immune complexes in the circulation or in the extracellular fluids of the host can induce tissue injuries upon further reaction with plasma factors and cells (*Cochrane* et al. 1959; *Dixon* 1963; *Wilson* and *Dixon* 1976; *Wilson* 1981). The Arthus reaction, an acute necrotizing vasculitis, is the simplest of these (*Arthus* and *Breton* 1903). The lesion develops when relatively large amounts of antigen-antibody complex form in extravascular spaces. A localized lesion around the vessel walls is produced if one of the reactants, either antigen or antibody, is present in the circulation and the other is injected locally. Initially, antigen and antibody diffuse toward each other, creating immune complexes which can then become insoluble. After activation of complement, the reaction site becomes highly chemotactic for polymorphonuclear leukocytes (PMN). Within a few hours, PMN cells infiltrate the tissues, resulting in necrosis. The PMNs phagocytose the antigen-antibody complexes and take them away from the site of reaction. In vitro studies have shown that the PMNs are capable of rapidly degrading the ingested complexes.

A more complex situation is *acute serum sickness*, which can be induced in experimental animals or in humans by injection of foreign proteins. The classical animal model is that of the rabbit injected i.v. with a single dose (250 mg/kg body weight) of bovine serum albumin (BSA) (*Dixon* et al. 1958). In this model, the disease develops 12–14 days after injection, following the production of anti-BSA antibodies and the formation of immune complexes. Coincidental with the presence of antigen-antibody complexes in the circulation, there is a fall in the level of serum complement and the appearance of acute inflammatory lesions in the kidney, heart, arteries, and joints, closely resembling the lesions of acute glomerulonephritis, rheumatic fever, lupus erythematosus, polyarteritis nodosa, and rheumatoid arthritis. Antigen, host IgG, and host complement fractions, presumably as complexes, are present at the sites of tissue lesions. The prominent characteristics of the lesions are the increased vascular permeability, endothelial proliferation, and variable PMN infiltration. The initial step in serum sickness appears to be a liberation of vasoactive substances (*Cochrane* and *Dixon* 1976). Following this, complexes begin to deposit in vessel walls. The depositing complexes have a phlogogenic effect. The circulating and fixed phagocytes take up and rapidly degrade most of the immune complexes. In acute experimental serum sickness, only 0.003% of the injected antigen is deposited in the kidney during development of the glomerulonephritis, and this antigen is eliminated from the kidney with a half time of 10 days.

In order to more closely mimic the conditions which likely occur in human pathology, experimental conditions were designed to keep small amounts of such immune complexes present in the circulation for long periods of time. This produces chronic progressive disease which depends primarily on the relative amounts of circulating antigen and antibody (*Dixon* et al. 1961). After one or more months of daily injections of BSA into rabbits, and periods of one to several weeks during which circulating antigen-antibody complexes are formed, the animals develop a progressive glomerulonephritis. The most common and probably the earliest anatomic form of this disease is a membranous glomerulonephritis characterized by thickened glomerular capillary basement membranes with little or no endothelial proliferation. Antigen, host IgG, and complement fractions are present in the thickened basement

membranes (*Dixon* et al. 1961; *Andres* et al. 1962; *Kniker* and *Cochrane* 1968). At this site, the tissue deposits persist for as long as 1 year after cessation of antigen injection.

2 Detection and Isolation of Immune Complexes

Two main approaches have been used in order to demonstrate the presence of immune complexes in human diseases: the analysis of tissue specimens and the serological analysis of samples from various biological fluids.

2.1 Detection of Immune Complexes in Tissues

Studies of tissue by conventional histological techniques and by electron microscopy may suggest involvement of immune complexes in the observed lesions on the basis of similarities to lesions induced experimentally by immune complexes. Immune-complex glomerulonephritis has been well defined by this approach. Immunohistochemical techniques allow a more direct demonstration of Ig deposits associated with complement components and some identified antigens in a pattern suggestive of the presence of immune complexes (*Wilson* 1981). Such techniques have been applied extensively to the investigation of many tissues and to the demonstration of immune-complex-like material within PMN in the circulation (*Steffelaar* et al. 1976) or in extravascular sites of inflammation (*Britton* and *Schur* 1971).

By a variety of elution procedures, immune complexes (antigens and specific antibodies) have been recovered from various tissues, predominantly the kidney (*Oldstone* 1975, 1976; *Koffler* et al. 1971; *Poskitt* et al. 1974; *Lambert* and *Dixon* 1968; *Costanza* et al. 1973; *Weksler* 1974; *Lewis* et al. 1971). The technique used for eluting immune complexes from the kidney has been examined comprehensively by *Woodroffe* and *Wilson* (1977) and by *Bartolotti* (1977). The elution of antibodies by low pH buffers or by destruction of a suspected complex-associated antigen (e.g., DNA by the specific enzyme DNase) allows identification of the components of tissue-localized complexes (*Koffler* et al. 1967; *Lambert* and *Dixon* 1968). However, eluted antibodies always represent a selected fraction of the complexed molecules and the elution procedures alter some antibodies more than others, particularly IgM antibodies. The total amount of eluted Ig must have a quantitative relationship to specific antibodies in order to evaluate their relative involvement in the deposited complexes.

The number of IgG-positive PMN and lymphocytes is increased and high levels of immune complexes are found in the blood of persons with certain viral diseases, such as dengue fever (*Theofilopoulos* and *Dixon* 1979). High percentages of Ig-positive lymphocytes are also found in patients with autoimmune diseases (*Winchester* et al. 1974). In these diseases, Ig on lymphocytes may represent either antilymphocyte antibody or a component of a bound complex. Moreover, in cancer patients, tumor antigen-antibody immune complexes may be adsorbed onto leukocytes (*Bansal* et al. 1976; *Hattler* and *Soehnlen* 1974). These cells could represent a convenient source for the isolation of immune complexes.

2.2 Detection of Immune Complexes in Biological Fluids

Studies of biological fluids provide evidence for the association of immune complexes with particular pathological conditions, either by the direct detection of immune complexes or by demonstration of serological changes which often are associated with the presence of immune complexes (*Zubler* and *Lambert* 1977, 1978; *WHO* 1977; *Lambert* and *Casali* 1978; *Casali* et al. 1979; *Casali* 1980; *Theofilopoulos* and *Dixon* 1979, 1980). The methods for the direct detection of immune complexes can be separated into two main groups. On the one hand, some methods allow for selective detection of immune complexes involving one given antigen through the discrimination between free and antibody-bound antigens. They represent *antigen-specific* methods. On the other hand, some methods have been devised in order to detect immune complexes independently of the nature of the antigen involved in the formation of these immune complexes. They represent *antigen-nonspecific* methods.

2.2.1 Antigen-Specific Methods

In particular clinical conditions, it may be useful to study whether a known antigen is involved in immune complexes. By using the appropriate methodology, one can distinguish between free antigen molecules and those specifically bound to Igs. In the case of particulate antigen, morphological analysis has been applied. The electron microscopic detection of aggregated Dane (hepatitis B virus) particles seems suggestive of their involvement in immune complexes (*Almeida* and *Wadsworth* 1969). Physicochemical methods have also been used in combination with the detection of known antigens, e.g., neuroblastoma-specific immune complexes have been detected in sera by radioimmunocounterelectrophoresis (*Jose* and *Seshadri* 1974). The differentiation between free and complex-bound antigen can be further achieved by measuring the amount of antigen which is removed from a biological sample when the host Igs are specifically precipitated or absorbed, e.g., the infectivity of sera from mice infected with LDV or LCMV decreases strikingly after the precipitation of Igs (*Notkins* et al. 1966; *Oldstone* and *Dixon* 1971a). Coprecipitation of Igs has also been applied to the detection of DNA-anti-DNA complexes following incubation of serum samples with radiolabeled actinomycin D, which binds as a marker to the DNA molecules (*Izui* et al. 1977). With respect to the multiplicity of antigen-antibody systems possibly involved in the in vivo formation of immune complexes, it is unlikely that such antigen-specific methods would be routinely used in clinical situations.

2.2.2 Antigen-Nonspecific Methods

Antigen-nonspecific methods are based on the distinct properties of complexed Ig as compared with free Ig molecules. The main properties which can be used are the physical changes due to complex formation and the biological activities of immune complexes, such as complement fixation or binding to cell membranes. These methods will detect nonspecifically aggregated Ig as well as immune complexes.

2.2.2.1 Methods Based on the Physical Properties of Immune Complexes

The formation of an immune complex leads to the occurrence of macromolecular structures with increased molecular size, as well as changes of the surface properties, solubility, and electric charge as compared with the corresponding free antigen and antibody. The extent of these changes will depend on the nature and concentration of each immunological constituent of the complex. For example, the *electrical charge* of an immune complex may differ from that of the corresponding free antibody molecule; thus, complexed IgG may be found to exhibit an abnormally increased affinity for anion-exchange resins (e.g., DEAE-cellulose) (*Fox* et al. 1974) or an altered electrophoretic mobility (*Grubb* 1975). Since the electrical charge of an immune complex is also highly dependent on the nature of the antigen involved, such an approach is useful as a discriminatory means for antigen-specific methods (*Teppo* et al. 1976) in a known antigen-antibody system.

Analytical ultracentrifugation has been applied to the detection of immune complexes in various biological fluids by the demonstration of an abnormal level of some material with a relatively high sedimentation velocity. However, the exclusive use of analytical ultracentrifugation does not allow the conclusion that immune complexes are present, since other materials of high molecular weight may also appear in large amounts in some biological samples. The sensitivity of this method is low. The use of preparative methods for separation of macromolecular aggregates, such as *sucrose gradient ultracentrifugation, gel filtration,* or *selective ultrafiltration* allows for the combination of physical analysis with the immunological and biological characterization of the various fractions. All of these methods, are based on a separation of immune complexes according to molecular size and are usually limited by contamination of the macromolecular fraction by monomeric immunoglobulins or unbound complement components. These preparative methods can represent an important step in the purification of immune complexes. They have been successfully applied to analytical studies of many clinical conditions, including measles (*Myllyla* et al. 1971). and they can be used in combination with added labeled markers for immune complexes, such as C1q or antiglobulins. One drawback of these techniques is that they are time-consuming and unsuitable for routine use.

Material with a decreased solubility under well-defined conditions (e.g., temperature, solubilizing medium) is found frequently in biological fluid containing immune complexes. Therefore, the occurrence of an abnormal precipitation of serum proteins in a serum sample under certain conditions may suggest the presence of immune complexes. The simplest procedure is precipitation at low temperatures. Cryoglobulins may represent a particular type of immune complex, but monoclonal Igs or other proteins may also be involved. Immunochemical analysis of the precipitates is required before considering cryoglobulins as possible immune complexes (*Cream* 1977). Certain media which can be used to separate serum proteins according to their physical properties can also be used to precipitate immune complexes and serum macromolecules under conditions in which free Ig molecules would remain soluble. The addition to serum of *polyethylene glycol* (PEG, mol. wt. 6000), an uncharged linear polymer, results in precipitation of proteins proportional to the concentration of the PEG (*Zubler* et al. 1976, 1977). The extent of precipitation of a protein is generally also proportional to its molecular size, so that at low concentrations of PEG, high molecular

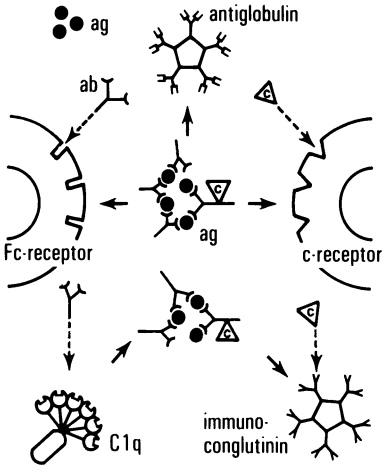

Fig. 5. Main cellular and humoral receptors by which an antigen (*ag*)-nonspecific recognition of immune complexes occurs. In the aggregated form in the complex, antibodies (*ab*) and complement (*C*) components usually exhibit a much higher avidity than the corresponding free molecules for Fc and complement receptors

weight proteins and immune complexes are preferentially precipitated. The demonstration of complement components, particularly C1q, C1r, C1s, C4, and C3, in such PEG precipitates suggests in vivo binding of complement to macromolecular complexes (*Casali* and *Lambert* 1979).

2.2.2.2 Methods Based on the Biological Properties of Immune Complexes

Several methods based on biological recognition of humoral or cellular receptors (Fig. 5) by soluble immune complexes are now available. This subject has recently been extensively reviewed (see Sect. 2.2). Some methods are based on the binding of complexes to free radiolabeled recognition units (e.g., [^{125}I]C1q or [^{125}I] bovine conglutinin) and the subsequent partition of bound recognition units from their unbound counterparts under appropriate physicochemical conditions, e.g., in the presence of PEG. In other methods, immune complexes bound to solid-phase receptors, either on cells or on a plastic support, are detected using radio- or enzyme-labeled probes such as anti-Ig antibodies or staphylococcal protein A. Competition methods can involve either free or bound receptors. In general, methods for detection of immune complexes, which are summarized in Table 1, can be divided into four groups.

Table 1. Antigen-nonspecific methods for the detection of soluble immune complexes based on the binding of biological recognition units

	Method	References
1. Recognition of immune complexes in cell-free systems		
1.1 Interaction with complement		
1.1.1 Complement and C1q deviation tests		
Measurement of anti-complement activity		*Nielsen* and *Svehag* (1976); *Johnson* et al. (1975)
C1q deviation (using sensitized erythrocytes)	RIA	*Sobel* et al. (1975)

Table 1. (continued)

	Method	References
C1q inhibition assay (using IgG-coated Sepharose particles)	RIA	*Gabriel* and *Agnello* (1977)
C1q latex agglutination inhibition assay		*Lurhuma* et al. (1976)
1.1.2 Direct measurement of C1q–immune complex interaction		
C1q agarose precipitation test		*Agnello* et al. (1970)
C1q-binding test	RIA	*Zubler* and *Lambert* (1976); *June* et al. (1979)
Solid-phase C1q-binding tests	RIA	*Hay* et al. (1976); *Ahlstedt* et al. (1976)
1.1.3 Binding of in vivo complement-reacted immune complexes to conglutinin or anti-C3 antibody		
Solid-phase conglutinin-binding tests	RIA/ELISA	*Casali* et al. (1977, 1978)
	RIA	*Eisenberg* et al. (1977)
	ELISA	*Manca* et al. (1980)
Conglutinin-binding test	RIA	*Macanovic* and *Lachmann* (1979)
Solid-phase anti-C3-binding test	RIA	*Pereira* et al. (1980)
1.2 Interaction with antiglobulin: monoclonal (mRF) or polyclonal (pRF) rheumatoid factors or anti-antibodies		
mRF agarose precipitation test		*Winchester* et al. (1971)
Solid-phase mRF inhibition assay	RIA	*Luthra* et al. (1975)
pRF inhibition assay (using soluble aggregated IgG and coprecipitation with anti-IgM)	RIA	*Cowdery* et al. (1975)
mRF inhibition assay (using IgG-coated Sepharose particles)	RIA	*Gabriel* and *Agnello* (1977)
pRF latex agglutination inhibition assay		*Lurhuma* et al. (1976)
Anti-antibody inhibition assay		*Kano* et al. (1978)
2. Recognition of immune complexes by receptors on cell surfaces		
2.1 Interaction with complement receptors on cells		
Raji cell assay	RIA	*Theofilopoulos* and *Dixon* (1976)
Lymphocyte complement–rosette formation inhibition test		*Smith* et al. (1975)
2.2 Interaction with Fc receptors on cells		
Platelet aggregation test		*Myllyla* (1973)
Inhibition of antibody-dependent cell-mediated cytotoxicity	RIA	*Thompson* et al. (1973)
Macrophage uptake inhibition test	RIA	*Mohammed* et al. (1977)
Neutrophil inhibition test		*Lambert* et al. (1978)
Neutrophil-dependent red cell browning test		*Lambert* et al. (1978)
Detection of immunoglobulin phagocytosis by neutrophils		*Steffelaar* et al. (1977)
Lymphocyte Fc rosettes formation inhibition test		*Morito* et al. (1976)

RIA, radioisotope assay; ELISA, enzyme-linked immunosorbant assay
References including updated protocols are indicated

In the first group of methods, reactivity of immune complexes with complement components is measured. The methods used include inhibition assays, such as the C1q deviation assay and the C1q latex agglutination test, and direct binding assays, such as the C1q solid-phase assay. The inhibition assays are limited by their high sensitivity to substances other than immune complexes. Indeed, the reaction of C3bi-coated immune complexes with bovine conglutinin or rabbit $F(ab')_2$ anti-human C3 has been applied to the detection of immune complexes in order to avoid interference from substances reacting with C1q.

The second group of methods is based on the reaction of anti-Ig antibodies with immune complexes. Several types of antiglobulins have been used, including monoclonal rheumatoid factor, polyclonal rheumatoid factor, anti-antibodies, and low-avidity heterologous antiglobulins. These tests are usually influenced by the concentration of IgG in the sample to be tested and they often suffer from interference due to the presence of rheumatoid factor in the sample.

The third group of methods is based on the reactivity of immune complexes with complement receptors on cells. The most familiar test in this group is the Raji cell radioassay using a lymphoblastoid cell line bearing C1q, C3, C4, and IgG Fc receptors as recognition units for immune complexes. Such methods may be influenced by the presence of anti-lymphocyte antibodies or of antibodies to other cell membrane antigens.

The fourth group of methods is based on the reactivity of immune complexes with Fc receptors on cells. Tests have been developed using a variety of cells including PMN, B lymphocytes, macrophages, and platelets. They are all functional assays and thus dependent on the integrity of the cells used. Therefore they may suffer from interference by cytotoxic factors in the tested samples, and they are influenced by the presence of rheumatoid factors. Some tests, such as the killer-cell inhibition assay, are also influenced by the IgG concentration in the sample.

In view of the variety of tests available for the detection of immune complexes, certain criteria are needed to define the ideal test system. Such a test should be (a) sufficiently sensitive; (b) relatively specific, but having a wide spectrum of activity for immune complexes; (c) reproducible; (d) relatively simple; and (e) it should not require heat-inactivation or absorption of the sample before testing. Among the tests available, at least seven seem to fill most of these criteria: the C1q-binding test, the C1q solid-phase assay, the solid-phase conglutinin binding assay, the anti-C3 assay, the Raji cell radioassay, the monoclonal rheumatoid factor (RF) inhibition test, and the platelet aggregation test. All of these tests show a similar range of sensitivity, but the most sensitive for aggregated Ig are the Raji cell assay and the solid-phase conglutinin binding assay. None of the tests requires heat-inactivation or absorption of the samples, and those based on the solid-phase principle can be modified to detect any specific kind of Ig isotype involved in the immune complexes. However, immune complexes containing IgE antibodies cannot be detected by any method presently described.

2.3 Indirect Evidence for the Presence of Immune Complexes

Since most biological effects of immune complexes are mediated through activation of the complement system, the involvement of complement should be regarded

as an important feature, closely associated with the pathological expression of the formation of immune complexes. The analysis of complement involvement can be carried out in serum and in extravascular fluids either by measurement of the hemolytic activity, by immunochemical evaluation of the concentration of the complement components, or by evaluation of their catabolism through turnover studies or quantitation of complement breakdown products. In acute inflammatory states, such as in viral diseases, the hypercatabolism of complement components can be masked by an increased synthesis, and static measurements do not strictly reflect the activation of the system. Although turnover studies using labeled components have occasionally been performed, such methodology is hampered by the difficulty in avoiding functional alterations of the complement proteins during the labeling procedure. An alternative approach is the measurement of the breakdown products of C3, C4, or factor B, which are released from the native molecules during the activation process. This has been achieved by immunoelectrophoresis (*Zvaifler* 1969, 1973) or by immunochemical quantitation, using radial immunodiffusion (*Perrin* et al. 1975). An important limitation of this approach is that the complement system may be activated by substances unrelated to immune complexes. In particular, lipopolysaccharides from several types of bacteria can efficiently consume complement by triggering the alternative and/or the classical pathways (*Mergenhagen* et al. 1973; *Loos* et al. 1974; *Winkelstein* et al. 1976; *Morrison* and *Kline* 1977).

2.4 Purification of Immune Complexes from Biological Fluids

Because immune complexes deposited in tissues may represent only a particular and/or minor component of the total immune complexes, the isolation of purified immune complexes from various biological fluids is an important step in the identification of their components. Certain optimal requirements for the isolation of all types of complexes can be defined. First, the physicochemical conditions of the procedure should not favor dissociation of the complex, and the use of low pH, high salt concentration, or extensive dilution should be avoided. Secondly, the procedure should have a relatively high degree of specificity, since immune complexes are usually present in serum at low concentrations, and contamination by other serum proteins can occur. Thirdly, the efficiency of the procedure must be sufficient to permit the recovery of the relatively large amounts of complexes needed for characterization.

Preliminary concentration of immune complexes from biological fluids can be attempted by precipitation with ammonium sulphate or PEG, gel filtration, and preparative ultracentrifugation in sucrose density gradients (described in Sect. 2.2.2). Staphylococcal protein A, which binds monomeric as well as complexed IgG, has also been used to concentrate complexes from serum (*Chenais* et al. 1977). Other approaches for concentration of immune complexes are based on their reactivity with biological receptors. Complement-reacting immune complexes have been isolated predominantly by such procedures. C1q coupled to Sepharose has been used as a receptor for C1q-binding complexes (*Svehag* and *Burger* 1976), rheumatoid factor coupled to Sepharose for complexed IgG (*Gilead* et al. 1981), and Raji cells for complexes containing fixed C3 (*Theofilopoulos* et al. 1978). In some cases, the nature of the complexed antigen allows the use of particular binding substrates, e.g., lectin

used for the binding of viral glycoproteins. Concanavalin A-sepharose has been used (*Heimer* and *Klein* 1976) to separate immune complexes from the sera of patients with Epstein-Barr virus-related cancer (e.g., Burkitt's lymphoma and nasopharyngeal carcinoma).

Bovine conglutinin (K) or human C1q have been used as biological receptors on biologically inert solid-phase matrices for the purification of soluble immune complexes from macromolecular fractions (*Casali* and *Lambert* 1979). It is worthwhile to outline the procedure for this efficient and flexible approach. Serum is first freed of lipid by treatment with liquified trichlorotrifluoroethane (Frigen), and a macromolecular fraction is then precipitated with PEG. The precipitate is solubilized and then absorbed onto a column of polymethylmethacrylate (PMMA) beads coated with K or C1q. C1q-fixing and complement-reacted immune complexes bind efficiently to C1q or K coupled to PMMA beads as solid-phase support. The column is washed and the complexes are eluted using 0.02 *M* ethylenediaminetetraacetate (EDTA), Tris-HCl buffer, pH 7.2 (for K columns) or 0.5 *M* NaCl, Tris-HCl buffer, pH 7.2 (for C1q columns).

Immune complexes formed in vitro were used for testing the solid-phase isolation procedure. The complexes, prepared in the presence of fresh normal human serum (NHS) and at modest concentrations of antibody (60–80 μg/ml), are perfectly soluble even when the antigen-antibody ratio was close to equivalence, and they remained soluble for a much longer period of time than that required for the experiments. This reflects the "solubilization" effect due to the binding of complement fractions to antibody molecules, as demonstrated by *Czop* and *Nussenzweig* (1976) (see Sect. 1.3.1 and Fig. 3). Conversely, immune complexes formed under the same concentrations of antigen and antibody, but in the absence of complement, exhibit a strong tendency to precipitate. However, such a situation is probably infrequent in vivo, and we consider the soluble immune complexes formed in vitro in the presence of complement as a better model for in vivo studies.

Solid-phase K and C1q bound [^{125}I]BSA-anti-BSA and [^{125}I]hepatitis B surface antigen (HB_sAg)-anti-HB_sAg complexes almost to the same extent at different antigen-antibody ratios, indicating that the capacity of complexes involving BSA or HB_sAg to bind C1q parallels their ability to activate the complement sequence, and particularly to bind C3. For other types of immune complexes, there could be dissociation between their C1q and complement-fixing abilities, as has been observed for [^{125}I]tetanus toxoid–anti-tetanus toxoid complexes, depending on he relative affinity of the antigen itself for C1q (*Casali* and *Lambert* 1979). Soluble [^{125}I]BSA-anti-BSA, [^{125}I]tetanus toxoid–anti-tetanus toxoid, and [^{125}I]HB_sAg–anti-HB_sAg complexes made in vitro in the presence of fresh NHS were eluted in purified form from solid-phase K or C1q. The isolated complexes were shown to contain antigen, antibody, C1q, C1r, C1s, and C3. When NHS was submitted to such a procedure, no detectable protein was present in the final eluted fraction. Immune complexes formed in vivo in the serum of patient with disseminated leishmaniasis were also purified on a K column. The isolated material was found to contain IgM, IgG, C1q, C1r, C1s, C3c, and C3d (Figs. 6 and 7). The purified complexes dissociated at acid pH and were found to contain anti-IgG and anti-leishmania antibodies (Fig. 8).

The combination of a physicochemical step (e.g., PEG precipitation), as a preparative procedure to concentrate soluble immune complexes, with a biological step

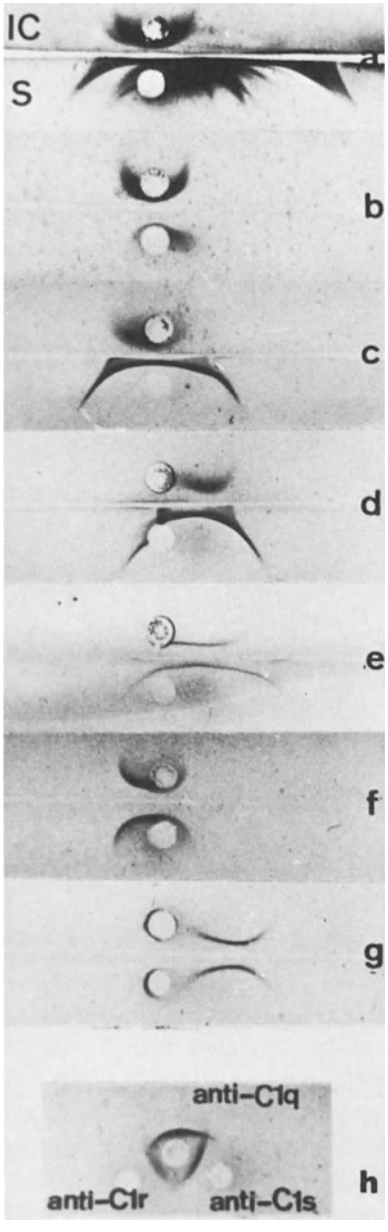

Fig. 6. Analysis of immune complexes purified from serum of patient with kala-azar by polyethylene glycol concentration and adsorption to solid-phase conglutinin. Immunoelectrophoresis (panels *a–g*) *upper wells*, eluate from solid-phase conglutinin; *lower wells*, normal humal serum (NHS); troughs, (*a*) anti-NHS, (*b*) anti-IgM, (*c*) anti-IgG, (*d*) anti-βIA (C3c), (*e*) anti-α_2d (C3d), (*f*) anti-C1q, (*g*) anti-C1s. Anode was on the right. *IC* = immune complex; *S* = normal human serum. Ouchterlony (*h*): *centre well*, eluate from solid-phase conglutinin. (*Casali* and *Lambert* 1979)

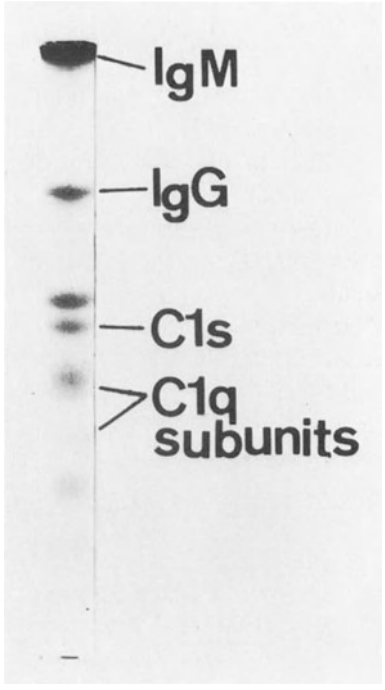

Fig. 7. SDS-PAGE analysis of immune complexes purified from patients with kala-azar. Sample to be analyzed was diluted in 2% SDS, 0.05 *M* phosphate, pH 7.0, heated at 56 °C for 30 min and then applied to a 5% gel. (*Casali* and *Lambert* 1979)

(binding to solid-phase K or C1q) represents an efficient procedure for obtaining complement-reacted immune complexes from biological fluids in a purified form and in amounts that allow for their characterization. Bovine K can be easily prepared in pure form using a recently described method (*Maire* et al. 1981). The procedure outlined here offers certain advantages. The bovine K is highly efficient in binding complement-reacted immune complexes in the presence of Ca^{++} at neutral pH, and release of the complexes can be induced without modification of the ionic strength or pH by use of a buffer containing 0.02 *M* EDTA. In addition, PMMA is a biologically

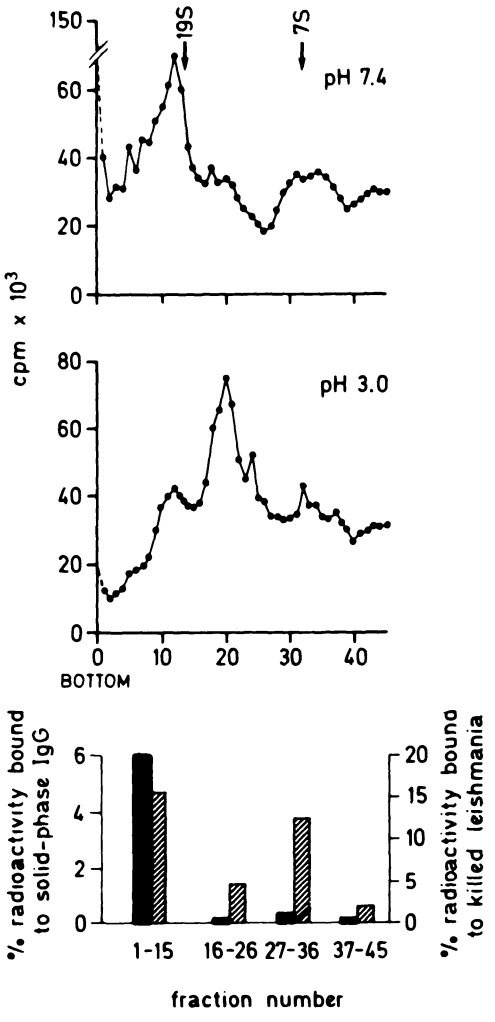

Fig. 8. Ultracentrifugation of the immune complexes purified from the serum of a patient with kala-azar. The purified complexes were labeled with [^{125}I] using the lactoperoxidase method and then applied to a 10%–40% sucrose density gradient either at pH 7.4 or at pH 3.0. *Solid line*, radioactivity of the collected fractions. *Broken line*, radioactivity stuck to the bottom of the tube. The material fractionated at pH 3.0, pooled in four fractions, was tested for anti-Ig (*solid columns*) and anti-Leishmania (*hatched columns*) activity. Anti-IgG activity was assessed using solid-phase human IgG, and anti-Leishmania activity using heat-killed Leishmania tropica. (*Casali* and *Lambert* 1979)

inert material which can be efficiently washed. Other types of immunoabsorbent matrix, such as Sepharose, require the use of high ionic strength buffers in the washing steps to avoid nonspecific binding of protein. The procedure described here does not lead to detectable contamination of eluted fractions by serum proteins. The successful purification of complexes formed in vivo from the serum of a patient with kala-azar demonstrated that this can be a useful new approach to study diseases associated with circulating soluble immune complexes. It should be noted that in the case of kala-azar, various specificities of antibody (anti-IgG, anti-leishmania), a possible antigen (IgG), and bound complement components (C1q, C1r, C1s, C3), were identified in the isolated complexes.

The approach described here is currently used in our laboratory to purify immune complexes from the serum of BALB/c and C3H mice infected with LCMV. Purified complexes have been analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and discrete bands are transferred from the SDS gel to paper by the Western blot technique for further immunochemical characterization.

3 Role of Immune Complexes in Viral Infections

Immune complexes occur in most viral infections, both in natural and experimentally induced infections of animals, as well as in virus infections of man. Evidence for the participation of such complexes in viral diseases is based either on immunohistochemical investigations of the tissue lesions associated with infection or on investigations of serum and other biological fluids. Such complexes appear most relevant in diseases caused by infectious agents of low pathogenicity, in which the immune response of the host plays a major role in determining the pathological manifestations. With a few exceptions, immune complexes may be involved in pathogenesis when a relatively large number of infectious particles chronically persist. Indeed, most of the persistent virus infections are associated with virus-induced immune complex deposits and disease (*Oldstone* 1975). After initiation of the infection, viruses as replicating agents provide an important supply of antigenic material. After production of antiviral antibodies by the host and liberation of infectious viruses by the infected cells, immune complex formation results. Complement-dependent lysis of the virion can occur in some viral infections like Sindbis, measles, and herpesviruses in human serum (*Stollar* 1975; *Oldstone* 1975; *Cooper* et al. 1976; *Cooper* and *Welsh* 1979). This lysis results in the liberation of internal viral antigens, which may be involved in the formation of new immune complexes.

Immune complexes are also produced at the level of infected cells in many viral infections in man caused by budding viruses like measles, mumps, and herpesvirus. In these infections, viral antigens are expressed at the surface of the infected cells before virus assembly and budding through the plasma membrane. Antibodies reacting with viral antigens at the surface of the infected cells first cap these antigens (*Joseph* and *Oldstone* 1974; *Joseph* et al. 1975; *Lampert* et al. 1975) (Fig. 9). The

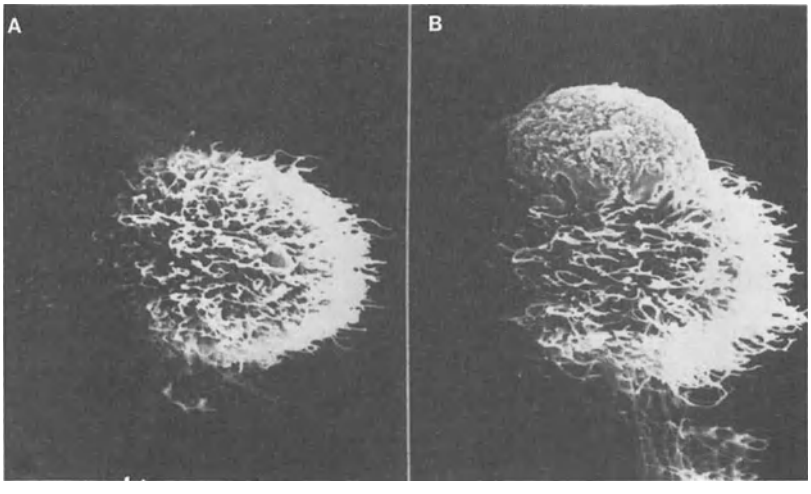

Fig. 9. Scanning electron micrographs of HeLa cells acutely infected with measles virus and expressing viral antigens on their surfaces. A) Cell not treated with antibodies to measles virus; B) Cell treated with multivalent antibody to measles virus at 37 °C. Note the redistribution of microvilli over one pole of the cell. (*Lampert* et al. 1975)

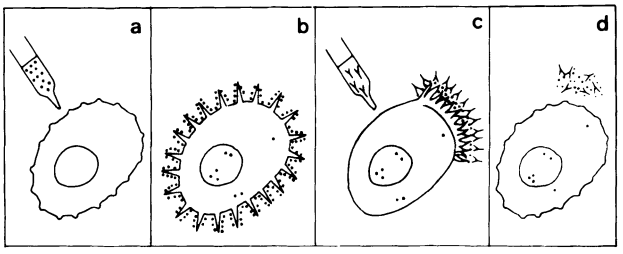

Fig. 10. Formation and release of immune complexes at the surface of HeLa cells infected with measles virus after incubation of the infected cells with human anti-measles virus antibodies. Cells were infected with measles virus (*a*), measles virus antigens are expressed at the surface of the infected cells 8 h after infection (*b*), antibodies are added in the medium and at 37 °C cap the measles virus antigen (*c*), and immune complexes are released into the medium (*d*)

antigen-antibody complexes are then partly ingested by the infected cells and partly released into the fluid phase. This has been demonstrated in vitro using measles-virus-infected HeLa cells and human anti-measles virus serum (*Perrin* and *Oldstone* 1977; *Oldstone* et al. 1980a) (Fig. 10). In this system, hemagglutinin, the major glycoprotein of measles virus, was found in complexes released into the fluid phase (Fig. 11). It is possible that, in vivo, the shedding of virus-antibody complexes originally bound to the plasma membrane would be a source of cell-free immune complexes. Virus-antibody complexes probably occur in most virus infections but are difficult to detect in acute infections, in which they are present in the circulation for only a short time. They are more frequently detected in chronic viral infections, in which persistent viral replication occurs in the presence of a continuing host response (*Tonietti* et al. 1970). In these circumstances, complexes can be detected in the circulation and in some tissues such as kidney, skin, small vessels, and choroid plexus.

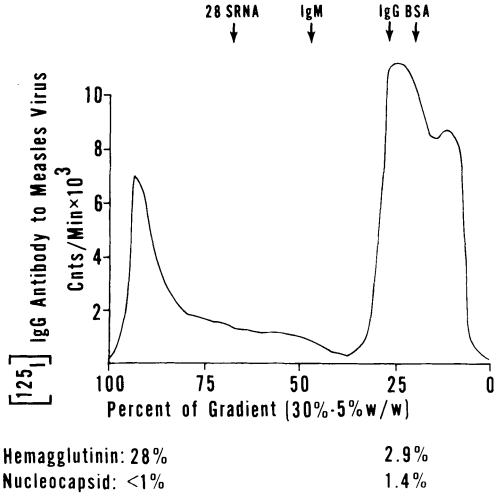

Fig. 11. Analysis of virus-[^{125}I]antibody complex shed off the surface of measles virus-infected cells after 3 h incubation. Complexes were analyzed for relative size by sucrose density centrifugation using known radiolabeled markers and for virus polypeptide composition by immunoprecipitation using antisera against known measles viral polypeptides, *BSA* (negative control) and *Ig* (positive control). The amount of hemagglutinin and nucleocapsid present in the complex was determined after subtracting the BSA counts. (*Oldstone* et al. 1976)

3.1 Animal Models

In the mouse, immune complexes occur during infection with several viruses including LCMV, LDV, retroviruses, polyoma virus, Coxsackie B virus and cytomegalovirus. Indeed, it is well established that the glomerulonephritis accompanying LCMV, LDV, and leukemia virus infection in mice (*Hirsch* et al. 1969), Aleutian disease in mink, and equine infectious anemia, is associated with deposition of virus-antibody complexes. A summary of the most widely known virus induced immune-complex diseases in mice is given in Table 2.

3.1.1 Immune Complexes in LCMV Infection of Mice

Mice infected in utero or at birth with LCMV harbor the virus for life (*Traub* 1936). This infection is not associated with any overt disease symptoms. In addition, many attempts to measure antibody in the sera of these virus-carrier mice by complement-fixation techniques have failed. On the basis of these observations, carrier mice were assumed to be immunologically tolerant to the virus.

Oldstone and *Dixon* (1969, 1970a, b) refuted this idea by showing that mice persistently infected with LCMV made specific antiviral Ig. The circulating antibody bound viral antigens to form virus-antibody complexes, which are found deposited in the renal glomeruli, arteries, and choroid plexus (reviewed by *Buchmeier* et al. 1980). The antibody deposited in the glomeruli could be eluted. The specificity of free anti-

Table 2. Evidence for virus-induced immune complex formation and disease in mice

	Presence of virus-antibody complexes		Recovery of specific antiviral antibodies from tissues
	Circulating	Deposited	
1. DNA viruses			
1.1 Herpesviruses			
Cytomegalovirus	unknown	+	+
1.2 Polyomavirus	unknown	+	+
2. RNA viruses			
2.1 Arenavirus			
Lymphocytic choriomeningitis virus	+	+	+
Tamiami virus	unknown	+	unknown
2.2 Retrovirus (leukovirus)			
Moloney leukemia virus	+	+	+
Moloney sarcoma virus	+	+	+
Spontaneous Gross leukemia virus	+	+	+
Friend leukemia virus	unknown	+	unknown
Rauscher leukemia virus	unknown	+	unknown
Rowson-Parr virus	unknown	+	unknown
2.3 Enterovirus			
Coxsackie virus B	unknown	+	unknown
2.4 Lactic dehydrogenase virus	+	+	+

body molecules could be demonstrated by absorption with virus-infected cell cultures (*Oldstone* and *Dixon* 1971a). Deposition of viral antigen-antibody complexes led to progressive development of glomerulonephritis and arteritis in virus-carrier mice (*Oldstone* and *Dixon* 1969, 1970a). Disease was found to be more severe in certain strains of mice (e.g., SWR/J) than in others (e.g., C3H). When disease severity was correlated with host antiviral antibody response, a positive correlation was found (*Oldstone* and *Dixon* 1970a).

It has been demonstrated that antibodies eluted from renal glomeruli of several strains of virus-carrier mice (*Buchmeier* and *Oldstone* 1978; *Oldstone* et al. 1980b) are directed against all of the LCMV polypeptides. Antibodies were eluted from the renal glomeruli of several strains of LCMV-carrier mice, as well as from pooled sera of AKR/J mice spontaneously infected with murine leukemia virus (*Oldstone* et al. 1974). The concentrations of IgG obtained from the LCMV-infected mice ranged from 5.7 μg per kidney for BALB/WEHI (nu/+) mice to 17.4 μg per kidney for SWR/J mice. The specificities of the recovered antibodies were assessed by a radioimmune precipitation assay (*Buchmeier* and *Oldstone* 1978) in which purified virus, metabolically radiolabeled with [^{35}S]methionine and [^{3}H]glucosamine, was reacted with the Ig eluted from the glomeruli. The antigen-antibody complexes were precipitated with antibody to mouse Ig and then analyzed by SDS-PAGE both for the amount of each label precipitated and for the presence of specific viral polypeptides. The specificity of the procedure was demonstrated by the failure of anti-LCMV antibody to precipitate Pichinde virus, an arenavirus not closely related to LCMV, and by showing that Ig

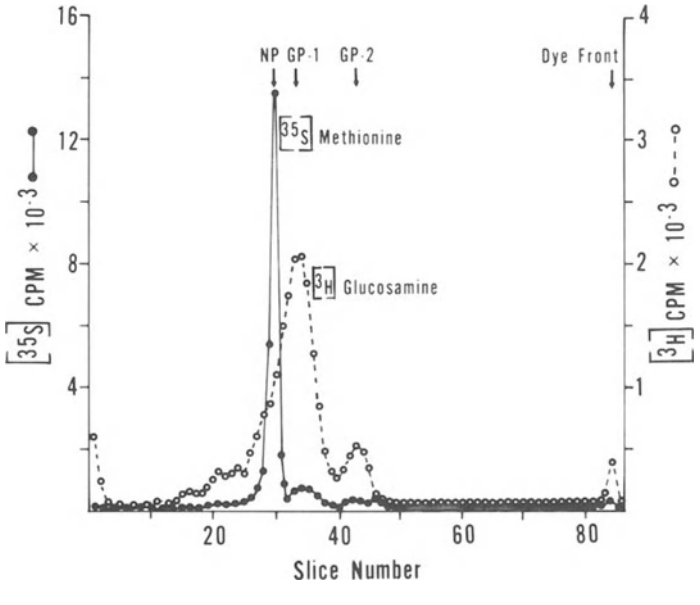

Fig. 12. Structural polypeptides of purified LCMV radiolabeled with [^{3}H]glucosamine (o---o) and [^{35}S]methionine (o——o). A preparation of purified virus was disrupted with SDS and β-mercaptoethanol and then electrophoresed on a 12.5% gel. The radioactivity in 1 mm slices was determined. Molecular weights of the viral polypeptides: nucleocapsid protein (*NP*), 63 000; glycopeptides (*GP-1*) and (*GP-2*), 35 000 and 44 000, respectively. (*Buchmeier* et al. 1978)

recovered from the kidneys of AKR/J mice (lacking antibodies to LCMV but having antibodies to murine leukemia virus) did not precipitate LCMV proteins. The structural polypeptides of LCMV and the polypeptide analysis of the immune precipitates by SDS-PAGE are shown in Figs. 12 and 13, respectively. The eluted antibodies had specificity for all three of the LCMV structural polypeptides. Indeed, there is no selective exclusion of a given virion antigen that could account for in vivo failure to eliminate the virus. The polypeptide specificities of serum antibodies were the same as those of antibodies recovered from the kidney. Further studies (*Oldstone* et al. 1980b) directly indicated that the quantitiy of this antibody is equivalent to that found in mice or guinea pigs hyperimmune to LCMV. The production of virus-specific antibody in carrier mice is thymus dependent (*Oldstone* et al. 1980b). Study of the

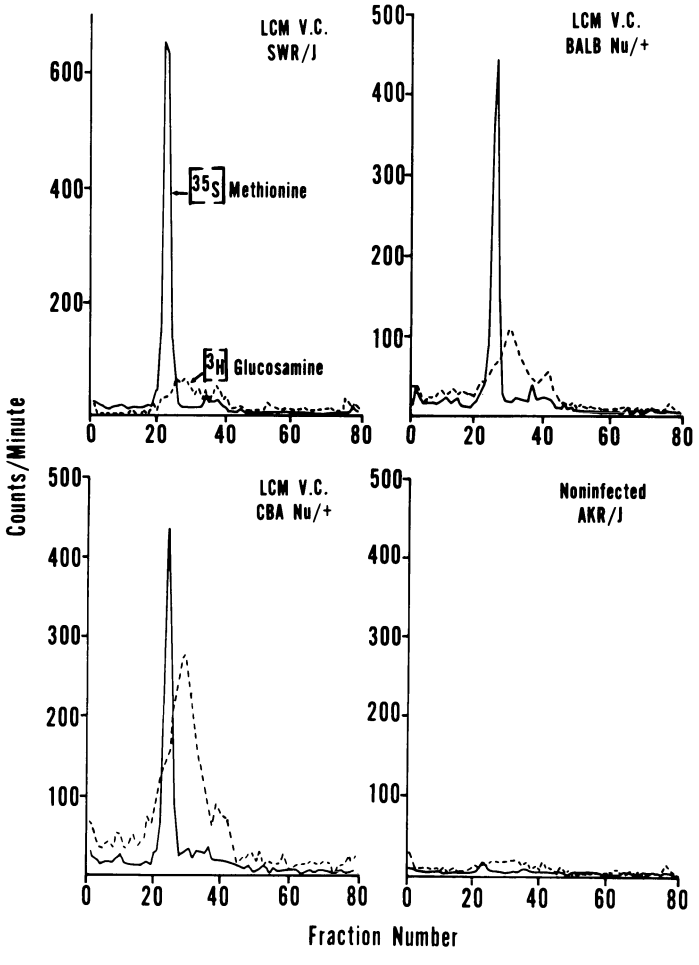

Fig. 13. Immune precipitation of the polypeptides of LCMV by Ig eluted from the kidneys of virus carrier (*LCM V.C.*) mice of three strains and control mice. Viral antigen was disrupted by incubation with Nonidet P-40 and RNase, and then allowed to react with the following quantities of eluted Ig: SWR/J, 6.5 μg; CBA nu/+, 2.8 μg; BALB nu/+, 4.2 μg; and AKR/J, 3.2 μg. The resultant immune precipitates were collected by precipitation with antibody to mouse Ig, washed, and analyzed by SDS-PAGE on 12.5% gels. (*Buchmeier* and *Oldstone* 1978)

antibody level in athymic nude (BALB/WEHI) mice and their thymus-sufficient littermates infected at birth with LCMV indicates that at 4 months of age, precipitating IgG antibody is present in all of the heterozygous mice but in none of the nude mice. Low levels of virus-neutralizing activity can be found, but not in all mouse sera. Using indirect immuno-flouorescence, specific antibodies that bind to the surface of virus-infected cells, but not mediating complement-dependent cytolysis, have been found in carrier mouse sera (*Doyle, Buchmeier* and *Oldstone* 1980, unpublished observations). These antibodies could act as blocking agents preventing immune recognition of virus-infected cells. LCMV-carrier mouse sera contained material banding at 9S–17S in analytical ultracentrifugation (*Oldstone* 1975). Considering the size of the virus particle, approximately 10 nm (*Murphy* et al. 1969, 1970), it is unlikely that this is the antigen of the complex that initiates tissue injury. In contrast, sera from mice persistently infected with LDV, which have high concentrations of antiviral antibody and infectious virus but do not develop severe immune-complex disease (*Notkins* et al. 1966; *Porter* and *Porter* 1971; *Oldstone* and *Dixon* 1971a), had only an 11S peak by ultracentrifugation analysis.

Persistent LCMV infection is a chronic disease characterized by glomerulonephritis, arteritis, focal hepatic necrosis, and widespread lymphocytic infiltration (*Oldstone* and *Dixon* 1969; *Accinni* et al. 1978). Deposition of immune complexes in tissues leads to chronic lesions. Foci of infiltrating cells contain abundant plasma cells (*Oldstone* and *Dixon* 1969, 1970b; *Accinni* et al. 1978). Immunofluorescent study of several tissues, especially the kidney, demonstrated the continued presence of viral antigen, suggesting that local immunoproliferation had occurred in response to the persistent presence of antigen (*Accinni* et al. 1978). Passive transfer of anti-LCMV antibodies into persistently infected mice leads to sequential infiltration of the kidney by PMNs, plasma cells, and lymphocytes (*Oldstone* and *Dixon* 1972).

Chronic LCMV-induced immune-complex disease is an ideal system in which to study the kinetics of viral immune complex formation and deposition, as well as the precise molecular composition of such complexes. After neonatal infection of SWR/J mice with LCMV (*Oldstone* et al. 1980b), C1q-binding activity has been found to appear in the serum by 4 weeks of age and to reach peak levels by 8–16 weeks of age. BALB/WEHI mice, which develop less severe glomerulonephritis, do not have high C1q-binding activity. Free antibody precipitating all of the LCMV polypeptides is found in the sera of both SWR/J and BALB/WEHI mice. However, virus-carrier SWR/J mice contain approximately fourfold more antibody to LCMV than do comparable BALB/WEHI mice. Further studies are needed to delineate the roles of antibody concentration, subclass, and affinity in the development of this immune-complex disease.

3.1.2 Immune Complexes in LDV Infection of Mice

Inoculation of mice with LDV results in persistent virus infection and elevated LDH enzyme activity (*Riley* and *Spackman* 1974). The virus multiplies in macrophages and produces widespread lymphoid hyperplasia and splenomegaly (*Riley* 1964; *Oldstone* 1975). As with LCMV infection, circulating immune complexes involving infectious particles are present throughout life. In virtually all 9-month-old mice of the SWR/J strain persistently infected with LCMV, complexes accumulate in heavy deposits in the

Table 3. Features of immune complexes associated with persistent infection of mice by LCMV or LDV

Infectious agent	Time after infection	Separation of infectious particles by rabbit anti-mouse Ig	C1q-binding material in serum	Immune complexes deposited in the kidney
LCMV	early (< 18 days)	+++	+++	+++
	late (> 18 days)	+++	+++	+++
LDV	early	+++	+++	+
	late	+++	○	○

glomeruli and are associated with severe nephritis (*Oldstone* and *Dixon* 1970a, b). In contrast, mice of the same strain and age persistently infected with LDV had fewer glomerular deposits, and clinical nephritis did not develop (*Porter* and *Porter* 1971; *Oldstone* and *Dixon* 1970a, b). Nearly 16 times more IgG was deposited in LCMV-infected kidneys than in kidneys of LDV-infected mice (*Oldstone* and *Dixon* 1971a). The characteristics of immune complexes and immune complex-associated pathology in mice infected with LCMV and LDV are illustrated in Table 3.

Mice persistently infected with LDV have circulating C1q-binding material within 10–18 days after infection, and the reduction of viral titers in the serum from these mice is always possible by treatment with anti-mouse Ig antibodies (*Notkins* et al. 1966; *Oldstone* and *Dixon* 1971a). Later in infection, C1q-binding material is not present in sera from these mice, yet the infectivity of the serum can be almost completely abrogated by treatment with anti-mouse Ig antibodies. This discrepancy between C1q-binding material and virus neutralization in serum by anti-mouse Ig antibodies has recently been investigated in detail (*McDonald* 1982). C1q-binding material could be isolated by affinity chromatography only from mice 10–18 days after infection with LDV. The isolated material contained IgG complexed with infectious particles. Conversely, later on in infection it was impossible to isolate C1q-binding material from the sera of these animals. A possible explanation for the data is that anti-LDV antibodies occurring late in the infection are of only selected isotypes, devoid of any C1-fixing activity.

Immune complexes with different biological activities, including complement fixation, could account for the different degrees of tissue injury found in mice infected with LCMV and LDV. LDV, and in many instances murine leukemia virus (MuLV), are associated with a mild asymptomatic glomerulonephritis. In contrast, the glomerulonephritis which accompanies LCMV infection is often severe.

3.2 Immune Complexes Associated with Viral Infection in Man

The wide application of recently developed methods for the detection of soluble immune complexes to the analysis of serum and various extravascular fluids indicates

that immune complexes occur in many human viral infections (*WHO* 1977; *Lambert* et al. 1978; *Casali* et al. 1979; *Theofilopoulos* and *Dixon* 1979, 1980).

3.2.1 Hepatitis B Virus Infection

Viral hepatitis can result from infection with type A hepatitis virus, resulting in epidemic hepatitis, type B hepatitis virus, resulting in serum hepatitis; or at least two other still unknown hepatitis-related viruses (non-A, non-B viruses) (*Zuckerman* 1982). Hepatitis B (HB) virus is a minimally cytopathic virus, the complete virus, a 43-nm particle (Dane particle) consisting of a 27-nm core containing circular double-stranded DNA and of an antigenically distinct coat (*Peterson* et al. 1975). Three antigen systems are now recognized: the HB_sAg (HB surface antigen or Australia antigen) present in the coat of the virus; the HB_cAg (HB core antigen), which is part of the nucleocapsid; and the HB_eAg, which is not yet characterized. HB antigens can be identified in the blood and in liver cells by electron microscopy and immunofluorescence. HB virus infections occur in most human populations but with a widely different prevalence and expression of disease. Indeed, responses to HB virus include asymptomatic infection with transient antigenemia and production of protective anti-HB_sAg-antibodies; asymptomatic infection with persistence of HB_sAg along with minimal humoral and cellular responses to HB_sAg resulting in a state of "chronic carrier" of HB virus; acute hepatitis with antigenemia and specific antibody during convalescence; and chronic hepatitis, which is associated with active humoral and cellular immune responses to the virus along with the presence of HB_sAg in the circulating blood and/or in the liver. Chronic hepatitis may develop from symptomatic or asymptomatic diseases. Antibodies to HB_sAg are usually present in patients with any of the disease manifestations. Immune complexes have been found at various stages of the disease by the [^{125}I]C1q-binding-activity test (*Nydegger* et al. 1974; *Thomas* et al. 1978), by the Raji cell test (*Theofilopoulos* et al. 1976a), or by immunoelectron microscopy (*Almeida* and *Wadsworth* 1969).

Using the C1q-binding test, immune complexes were detected in sera from patients with acute transient hepatitis and with chronic persistent hepatitis, but not in healthy carriers of HB_sAg (*Nydegger* et al. 1974; *Thomas* et al. 1978). There was no correlation between clinical status and serum C1q-binding activity in patients with acute hepatitis, but the C1q-binding activity declined as the circulating HB_sAg disappeared. In persons with chronic active hepatitis, the degree of C1q binding correlated well with the severity of disease (*Thomas* et al. 1978). Concentrations of various complement components decreased during acute hepatitis (*Kosmidis* and *Leader-Williams* 1972; *Thompson* et al. 1973; *Charlesworth* et al. 1977). Using the Raji cell assay, *Theofilopoulos* et al. (1976a) detected immune complexes in the sera of more than half of patients with acute serum hepatitis, even in those without detectable HB_sAg. In contrast, only 13% of asymptomatic carriers with circulating HB_sAg were positive. In the majority of these patients, the amount of immune complexes diminished markedly as the elevated serum enzyme levels fell during convalescence. Upon sucrose density fractionation, sera positive for immune complexes showed IgG, C3, and HB_sAg migrating together, whereas sera negative for immune complexes did not. When Raji cells were incubated with the contents of the gradient band containing IgG, C3, and HB_sAg, and then stained with fluorescein-conjugated antisera to HB_sAg, a fluores-

cence-positive reaction indicated the presence of this antigen on the cell surface, presumably as part of immune complexes. *Carella* et al. (1977), using PEG precipitation, also detected HB_sAg and specific antibody in the precipitates obtained from the sera of patients with acute or chronic hepatitis. Similar immune complexes have been detected in sera and cryoprecipitates from hepatitis patients by various other procedures (*Lurhuma* et al. 1976; *Digeon* et al. 1977, 1979; *Prince* and *Trepo* 1971; *McIntosh* et al. 1976; *Myllyla* 1973; *Madalinski* and *Bragiel* 1979; *Pernice* et al. 1979).

Infection with HB virus is sometimes characterized in its prodromal phase by a serum sickness-like syndrome, which could be due to circulating immune complexes. Indeed, immune complexes are associated with vasculitis and arthralgia (*Alpert* et al. 1971, 1972; *Sergent* et al. 1976), and complement-fixing HB_sAg-anti-HB_sAg antibody complexes are present in cryoprecipitates derived from sera of patients with such prodromic symptoms (*Wands* et al. 1975). It has also been shown that a high proportion of patients suffering from essential mixed cryoglobulinemia have in their cryoprecipitates either HB_sAg and/or anti-HB_sAg-antibody. In some of the cryoprecipitates, morphological structures corresponding to the HB virus particles or to Dane particles were also detected (*Levo* et al. 1977).

Several histological forms of glomerulonephritis accompany serum hepatitis and are typified by granular deposits of Ig and complement as well as electron-dense deposits along the glomerular basement membranes (*Brzosko* et al. 1974). Ig deposits have also been found along tubuli, suggesting additional extraglomerular deposition of immune complexes. In addition, HB_sAg or HB_eAg have been identified (*Brzosko* et al. 1974; *Knecht* and *Chisari* 1978; *Takekoshi* et al. 1979). These findings suggest that extrahepatic manifestations of HB infection are mediated by HB_sAg-anti-HB_sAg antibody complexes.

An association between HB_sAg and polyarteritis has been established (*Gocke* et al. 1970, 1971; *Kohler* 1975; *Duffy* et al. 1976), and immune complexes are present in sera and tissues from these patients (*Trepo* and *Thivolet* 1970; *Prince* and *Trepo* 1971; *Trepo* et al. 1974).

3.2.2 Dengue Hemorrhagic Fever

Dengue hemorrhagic fever (DHF) is caused by dengue virus, a subgroup of group B arboviruses (flaviviruses), which are presently divided into four distinct antigenic types. Following infection with dengue virus, high mortality is observed in infants and children in Southeast Asia (*Russel* 1971). Patients infected with dengue virus manifest mild symptoms or the more severe syndrome, DHF, sometimes accompanied by shock. The majority of people with DHF display evidence of previous dengue infection. It is suggested that virus multiplication can be amplified during a second infection by the following mechanism. Antibody produced on the first infection will bind but not neutralize dengue virus of another strain. The virus-antibody complexes will then bind to human monocytes through their Fc receptors, and the replication of the virus will be increased (*Russel* 1971). Indeed, it was found that immune complexes formed in vitro involving homotypic and heterotypic antibody to dengue-2 virus were more efficient in infecting macrophages than the virus alone, and that the enhanced infectivity was dependent on the Fc portion of the antibody (*Halstead* and *O'Rourke* 1977a, b). Dengue virus replicates in vitro not only in macrophages but also in antigen-

stimulated B cell blasts (*Theofilopoulos* et al. 1976b; *Halstead* et al. 1977) and endothelial cells (*Andrews* et al. 1978). Direct vascular injury can be produced by viral replication in endothelial cells. Activation of the complement system by both the classical and alternative pathways and intravascular coagulation, resulting in thrombocytopenia, reduction of coagulation factors, and presence of fibrinogen degradation products are characteristic of DHF and dengue shock (*Bokisch* et al. 1973). Immune complexes seem likely to be responsible for triggering these effector mechanisms. Indeed, immune complexes are present in the blood (*Theofilopoulos* et al. 1976a) and deposited in tissues and on the surfaces of lymphocytes (*Boonpucknavig* et al. 1976). A putative role for these immune complexes in this disease is suggested by the high correlation between C1q-binding activity in serum and severity of the hemorrhagic syndrome (*Sobel* and *Bokisch* 1975; *Ruangjirachuporn* et al. 1979).

3.2.3 Epstein-Barr Virus Infections

Epstein-Barr virus (EBV) is associated with three human diseases: infectous mononucleosis (IM), Burkitt's lymphoma, and nasopharyngeal carcinoma (*Henle* and *Henle* 1975). Circulating immune complexes have recently been found in the acute phase of IM (*Wands* et al. 1976; *Charlesworth* et al. 1978). In IM, autoantibodies with diverse specificities and antibodies directed against haptenic structures have been described (*McKensie* et al. 1976). Several mechanisms could be responsible for the appearance of autoantibodies in EBV and possibly in other viral infections: (a) a mitogenic effect of some viral constituent, inducing polyclonal B cell activation; (b) a release of sequestered antigens by the viral infection; (c) a virus-induced alteration of host cell membranes; and (d) direct or indirect alteration of immunoregulatory cells. Immune complexes have also been found in Burkitt's lymphoma and nasopharyngeal carcinoma (*Oldstone* et al. 1975a; *Heimer* and *Klein* 1976; *Lambert* et al. 1978) and immune complex deposits have been detected by immunofluorescence in the glomeruli of kidney biopsies of patients with Burkitt's lymphoma.

3.2.4 Cytomegalovirus Infections

Cytomegaloviruses (CMV) are the most frequent cause of congenital infections in man, occurring in 0.5%–2.4% of all live births (*Stagno* et al. 1977). CMV may also be acquired by an additional 3%–5% of neonates and young infants by exposure to maternal genital tract secretions, breast milk, and household sources. Overt disease occurs only in a small minority of infants infected in utero. However, significant numbers of those subclinically infected at birth manifest injury later in life. Persistent viral replication occurs in salivary gland and renal parenchyma (*Stagno* et al. 1975) as well as in other tissues not accessible to routine virological examination. The elicited specific humoral immune response is substantial and prolonged whether the infection is productive or latent (*Stagno* et al. 1977). Using a microcomplement consumption test and the Raji cell assay, immune-complex-like materials were detected in 35%–45% of sera from patients with congenital or postnatal cytomegalovirus infections. The presence of immune complexes was more frequent in children with severe intrauterine infection and, during the first year of life, paralleled the patterns of viral excretion and antibody titers. Circulating immune complexes appeared to be larger (18S–22S) in sick

infants, as opposed to asymptomatically infected infants in whom intermediate-size complexes (12S–16S) were found. In symptomatic patients with severe congenital cytomegalovirus infection, granular deposits of Ig and C3 were detected along the glomerular basement membrane.

3.2.5 Measles Virus Infection

Subacute sclerosing panencephalitis (SSPE) is a progressive neurological disease affecting children and adolescents. Patients with this disease have extremely high levels of antibody to measles virus in the serum and in the cerebrospinal fluid. The brain tissue contains inclusion bodies and nucleocapsid material identical to measles virus. Measles virus has been isolated from brain using cocultivation techniques and is thought to be the causative agent of SSPE (*Sever* and *Zeman* 1968). Immune complexes have been found in the circulation of these patients, but it is not known whether or not virus or viral antigens are involved in the complexes (*Dayan* and *Stokes* 1972; *Phillips* 1972; *Oldstone* et al. 1975b). Some investigators have found a serum factor that could block cell-mediated killing, macrophage inhibitory factor activity, and lymphocyte proliferation. This factor is precipitated by antibodies against IgG and C3, which suggests that it could be an immune complex (*Ahmeed* et al. 1974).

4 Summary and Conclusions

Immune complexes occur frequently during most viral infections in experimental animals and man. This evidence is based on the demonstration of immune complexes in blood and in tissues. The presence of soluble immune complexes has been established by physicochemical analysis of serum and by the recognition of biological effects largely specific for immune complexes.

The *biological properties of immune complexes* result mainly from the presentation of antigen and antibody molecules in an aggregated form, and therefore at a higher density than the corresponding free molecules. Aggregated antigens or antibodies are bound more avidly by cellular and humoral receptors than are their isolated counterparts. In vivo, the fate of immune complexes is directly dependent on the site of their formation, on their nature, as well as on their relative concentrations. Extracellular immune complexes can be formed in the circulation or in extravascular fluids, or in both compartments. Circulating large-size immune complexes are cleared rapidly from the circulation by mononuclear phagocytic cells. Small-size, particularly non-complement-fixing complexes are poorly cleared from the circulation and can be fixed in vessel walls or in filtering membranes such as those of the kidney and choroid plexus. Immune complexes formed in extravascular spaces are poorly cleared and may induce focal inflammation. *Tissue studies* may suggest the involvement of immune complexes in the observed lesions on the basis of similarities to lesions induced by immune complexes in experimental models. *Studies of biological fluids* can provide evidence for the presence of immune complexes, either by their direct detection or by demonstration of serological changes which are often associated with the presence of immune complexes.

The methods for the detection of immune complexes in biological fluids are either "antigen specific" or "antigen nonspecific". Antigen-nonspecific methods are based on the distinct properties, physical or biological, of complexed Ig molecules. Most of the work on soluble immune complexes in viral infections involves tissue studies and methods of detection based on biological recognition of immune complexes by humoral molecules or cellular receptors.

It appears that immune complexes occur in most viral infections, but their effects are variably expressed. The main pathological manifestations are directly related to the formation of immune complexes and to their localization in tissues. Under these conditions, the quantitation of circulating or extravascular immune complexes and the estimation of complement catabolism may be useful in monitoring the course of the disease. The formation of immune complexes may represent a secondary event during the course of an infection but may account for some of the primary manifestations, such as the serum sickness-like-syndrome seen in HB infections. Finally, immune complexes may occur in some viral infections without obvious direct pathological consequences.

The nature of the immune complexes occurring during viral diseases is often unknown. Evidence accumulated until now suggests that in infections of both experimental animals and man, at least a portion of the circulating complexes consists of infectious particles bound to specific antibodies. However, other antigen-antibody systems can be expected to be involved in the formation of such complexes, e.g., IgG-antiglobulin and/or IgG-anti-idiotypic antibody. The advent of new methodological approaches allowing the separation of immune complexes from biological fluids in pure form for their characterization should contribute to clarification of this matter.

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Immunoscintigraphy for the Detection of Human Carcinoma After Injection of Radiolabeled Monoclonal Anti-Carcinoembryonic Antigen Antibodies

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1 Introduction

Research on tumor localization by radiolabeled antibodies was initiated almost 30 years ago by *Pressman* and *Korngold* (1953), who showed that labeled antibodies against Wagner osteosarcoma were concentrated in vivo in these tumors. One of Pressman's major contributions in this field was to introduce the paired labeling method (*Pressman* et al. 1957), in which both antibodies and a control preparation of IgG, each labeled with a different isotope, are injected simultaneously into the same tumor-bearing animal. The measurement of radioactivity from each isotope in a dual channel scintillation counter allows one to distinguish the specific localization of antibodies in a tumor from the nonspecific accumulation of normal IgG, which is known to occur in the inflammatory and necrotic regions of a tumor (*Cerottini* and *Isliker* 1967). Using this method in golden hamsters bearing a human choriocarcinoma grafted into their cheek pouches, *Quinones* et al. (1971) showed that ^{125}I-labeled rabbit anti-human chorionic gonadotrophin (hCG) IgG antibody localized in the tumor. The control IgG, however, was also concentrated in the tumor, and only on day 4 after the injection was there was a significant, although moderate, increase in the concentration of relative to control IgG in the tumor.

In 1974, we introduced the model of nude mice bearing grafts of human colon carcinoma and the use of affinity-purified antibodies against a well-characterized antigen, such as carcinoembryonic antigen (CEA) (*Gold* and *Freedman* 1965). We showed that purified ^{131}I-labeled goat anti-CEA antibodies could reach up to nine times higher concentrations in the tumor than in the liver, whereas the concentration of normal IgG in the tumor was never higher than 2.3 times of that seen in the liver

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(*Mach* et al. 1974). We observed, however, great variations in the degree of specific tumor localization using the same preparation of labeled antibodies when colon carcinoma grafts derived from different donors were tested. This is probably due to the fact that human tumors keep their initial histologic properties and degree of differentiation after transplantation into nude mice, and these two factors appear to affect the ease with which circulating antibodies gain access to the CEA present in the tumors. The detection of ^{131}I-labeled antibodies in tumors by external scanning also gave variable results. With colon carcinoma grafts from some donors we obtained scans showing good tumor localization, whereas with colon carcinoma grafts from other donors the antibody uptake was not sufficient to give satisfactory scanning images. In this context we think that results in the nude mouse model are a good reflection of the clinical situation. Independently, *Goldenberg* et al. (1974) showed specific tumor localization and detection by external scanning with ^{131}I-labeled IgG fractions of anti-CEA serum using two human carcinomas which had been serially transplanted into hamsters for several years.

The first successful detection of carcinomas by external scanning in patients injected with purified ^{131}I-labeled goat anti-CEA antibodies was reported by *Goldenberg* et al. (1978). In their initial series of 18 patients they reported that scanning performed 48 h after injection detected almost all the CEA-producing cancers and gave no false positive results in patients without demonstrable tumors, or in patients with tumors apparently devoid of CEA. In contrast, our group had previously obtained negative results in a small series of 4 patients (*Mach* et al. 1978); and more recently in a series of 53 carcinoma patients injected with highly purified goat anti-CEA antibodies or fragments thereof, we obtained positive scans in only 22 patients (42%) with CEA-producing tumors (*Mach* et al. 1980a, b). Thus, our clinical results only partially confirmed those of *Goldenberg* et al. (1978; 1980). Our conclusion from this study was that this method of tumor detection is not yet clinically useful and that further research in specialized centers is necessary before it can be recommended as a routine clinical test.

The obvious advantages of monoclonal antibodies (Mabs) are their homogeneity and their specificity for the immunizing antigen. Another advantage of Mabs is that each Mab reacts with a single antigenic determinant. Thus, if this determinant is not repetitive on the recognized molecule, the Mab will not be able to form large immune complexes with the antigen. For instance, the seven anti-CEA Mabs that we have obtained were not individually able to precipitate CEA when tested by double diffusion in agar gels.

Anti-CEA Mabs were produced by the fusion of spleen cells from mice immunized with purified CEA (*Fritsche* and *Mach* 1977) and cells from the myeloma cell line P3-NSI/IAg4 (*Köhler* et al. 1976), using the method of *Köhler* and *Milstein* (1975). Out of the 400 hybrids obtained in a first series of seven fusions, two secreted antibodies reacting specifically with two different antigenic determinants present on the CEA molecule (*Accolla* et al. 1980). The specificity of the anti-CEA Mabs was demonstrated in an inhibition radioimmunoassay in which 1000 times more perchloric acid extract of normal colon or normal lung was necessary to give the same inhibition of CEA as the same extract from a colon carcinoma. In addition, the anti-CEA Mabs were not inhibited by cross-reaction of the "normal glycoprotein" (NGP) with CEA (*Mach* and *Pusztaszeri* 1972). The affinity constant of the best Mab (VII 23) (*Accolla*

et al. 1980), which we have used in immunoscintigraphy, was $Ko = 1.4 \times 10^8$ Mol^{-1}. In comparison, the average affinity of our immunoadsorbent-purified goat anti-CEA antibodies was only slightly higher $Ko = 2 \times 10^8$ Mol^{-1}. Anti-CEA Mabs were purified from ascitic fluid obtained from mice injected i.p. with $1-2 \times 10^7$ hybrid cells. Purification was achieved by precipitation from a 45% saturated ammonium sulfate solution followed by DEAE 52 ion-exchange chromatography. The specific antibodies were eluted with a gradient of slowly increasing molarity (starting with 0.01 *M*) phosphate buffer at a constant pH 8 (*Buchegger* et al. 1980). After labeling with [^{131}I] at 4 °C using the chloramine T method, the Mabs were filtered on a Sephadex G-200 column equilibrated in pyrogen-free 0.15 *M* saline. The 7S fraction selected for injection was tested for its binding to insolubilized CEA. The percentage of specific binding was 70% for Mab VII 23, its isotype was IgG.

2 Results

Twenty-eight patients with large-intestine carcinomas and two with pancreatic carcinomas were injected each with 0.3 mg ^{131}I-labeled anti-CEA Mabs with a radioactivity of 1.0–1.5 mCi. The labeled antibody was filtered through 0.22 μm Millipore filters and tested for sterility and for absence of pyrogenicity (in rabbits) before injection. The patient's premedication included Lugol 5% iodine solution, promethazine, and prednisolone, as previously described (*Mach* et al. 1980a, b). The patients had no previous history of allergy. They were also tested with an intracutaneous injection of normal mouse IgG and found to have no hypersensitivity to this protein. None of the patients showed any sign of discomfort during or after the injection of the labeled mouse antibodies. The patients were studied by external photoscanning 24, 38, 48, and 72 h after injection. An Elscint large-field camera with an LFC9 high-energy parallel-hole collimator was used. In 14 of the 28 patients (50%) a radioactive spot corresponding to the tumor was detected 36–48 h after injection. In 8 patients the scans were doubtful and in the remaining 8 patients they were entirely negative.

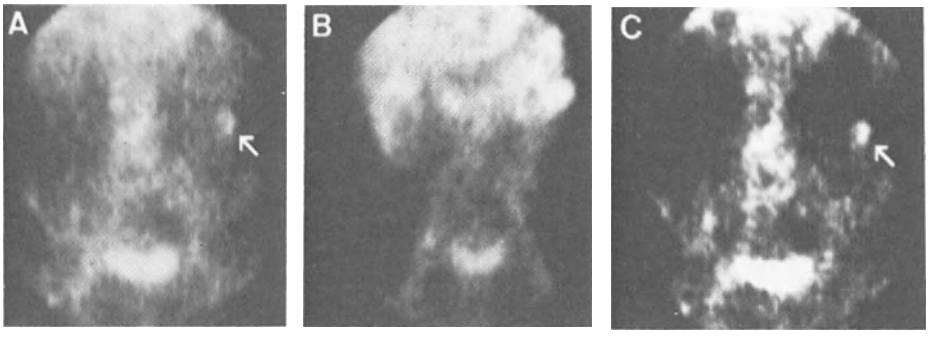

Fig. 1. External photoscannings of the abdomen and pelvis of a 75-year-old patient with a carcinoma of the left colon who was injected with 0.3 mg purified anti-CEA Mabs labeled with 1.5 mCi [^{131}I] 48 h before scanning and with 500 μCi ^{99m}Tc-labeled human serum albumin and 500 μCi free [^{99m}Tc04] 15 min before scanning. *Panel A* shows total [^{131}I] radioactivity, *panel B* [^{99m}Tc] radioactivity, and *panel C* [^{131}I] radioactivity after computerized subtraction of [^{99m}Tc] radioactivity. The radioactive spots in *panels A* and *C (arrows)* correspond to the tumor

An example of the scanning results obtained with a patient having a sigmoid carcinoma of 3.2 cm diameter is shown in Fig. 1. Panel A shows the total [^{131}I] radioactivity, panel B the [^{99m}Tc] radioactivity, and panel C the [^{131}I] radioactivity after subtraction of the [^{99m}Tc] radioactivity. The radioactive spots marked by the arrows correspond to the localization of the tumor. In addition, measurement of radioactivity in the tumor and adjacent tissues recovered after surgery showed that the concentration of labeled antibodies was 2.5–5.0 times higher in the tumor compared with that in the adjacent normal intestine.

In order to further evaluate the specificity of tumor localization by the paired labeling method, a few patients scheduled for tumor resection were injected simultaneously with 0.3 mg Mab VII 23 labeled with 1 mCi [^{131}I] and 0.3 mg normal mouse IgG labeled with 0.2 mCi [^{125}I]. The radioactivity of both isotopes was measured in the resected tumors and adjacent normal tissues, and the specificity of tumor localization was studied by differential radioactive analysis, as shown in Table 1. The "factors of radioactivity uptake" (indicated in parentheses in Table 1) ranged from 2.7 to 7.4 for Mab anti-CEA comparing tumor with normal mucosa. They are even higher when comparing the tumor with the intestinal wall stripped from the mucosa (normal serosa). In contrast, the same factors calculated for the normal IgG ranged between 0.8 and 1.6. Dividing the factors of uptake calculated for antibodies by those calculated for normal IgG, the "indices of specific tumor localization of antibodies" are obtained (underlined values in Table 1). The average of these indices for the four patients analyzed here is 4.3 (range 2.2–8.4).

3 Discussion

The idea of localizing an occult cancer by injecting a radiolabeled antibody is very attractive. For the specialist in nuclear medicine it represents a badly needed tool, while for the tumor immunologist it awakens the old dream of the "magic bullet" capable of delivering cytotoxic drugs (*Gregoriadis* 1981) or toxins (*Blythman* et al. 1981) to the hidden cancer cells. In the light of such high expectations, however, one must remain objective and realistic. We have shown that one anti-CEA Mab (Mab VII 23) can localize specifically in tumors, but the absolute amount of radioactivity detected in the tumor was still relatively low. Other anti-CEA Mabs should be tested, independently or as a mixture, provided that they react with different antigenic determinants on the CEA molecule.

In addition, the whole battery of Mabs against different tumor markers which have been reported over the last 3 years can be considered for use in immunoscintigraphy. However, before one considers injecting new Mabs into a patient, careful in vitro studies and nude mouse experiments should be performed to determine the percentage of the purified radiolabeled Mab which is able to bind to the tumor cells. Recently a Mab anti-human teratoma has been shown to localize in a human teratoma tumor grafted into immune-suppressed mice. An interesting antibody is the Mab anti-colon carcinoma 17-1A produced by the Koprowski group (*Herlyn* et al. 1980). Using this Mab we obtained encouraging results from immunoscintigraphy in nude mice and in a first series of carcinoma patients (*Mach* et al., to be published). We further suggest that any kind of tumor-specific Mab which is seriously considered for passive immuno-

Table 1. Specificity of tumor localization of monoclonal anti-CEA antibodies as compared with normal IgG

Case no.	Tumor site	Serum CEA (ng/ml)	Inj. to op. (days)	Tumor weight (g)	Material injected	Total Radio-activity in Tumor (nCi)	nCi/g Tumor	nCi/g Normal mucosa[b]	Tumor[a] Normal mucosa		nCi/g Normal serosa[c]	Tumor[a] Normal serosa		nCi/ml Serum	Tumor[a] Serum	
63	Tr. colon	1.2	6	15	Mab 23 ^{131}I	425	28.3	10.3	(2.7)	3.5	6.6	(4.3)	4.8	30.3	(0.9)	5.4
					Normal IgG ^{125}I	45	3.0	3.8	(0.8)		3.3	(0.9)		17.5	(0.17)	
67	Left colon	1.1	8	16	Mab 23 ^{131}I	196	12.3	4.6	(2.7)	2.2	2.5	(4.9)	3.0	17.7	(0.7)	3.3
					Normal IgG ^{125}I	50	3.1	2.5	(1.2)		1.9	(1.6)		14.8	(0.2)	
73	Left colon	2.7	4	28	Mab 23 ^{131}I	1052	38.4	11.3	(3.4)	2.4	7.8	(4.9)	3.8	45.4	(0.8)	3.8
					Normal IgG ^{125}I	77	2.8	2.0	(1.4)		2.1	(1.3)		13.3	(0.21)	
81	Sigmoid	150	5	50	F(ab')$_2$ 23 ^{131}I	897	17.9	2.4	(7.4)	4.6	1.8	(10.1)	6.7	7	(2.6)	8.4
					Normal IgG ^{125}I	184	3.7	2.3	(1.6)		2.4	(1.5)		12	(0.31)	

(Mach et al. 1981; reprinted with permission of *Immunology Today*)

[a] Factors of radioactivity uptake in tumor (indicated in parentheses) are obtained by dividing the concentration of radioactivity in tumor by the concentration of radioactivity in either normal mucosa, normal serosa, or serum. Specificity indices (underlined figures) are calculated by dividing the factor of radioactivity uptake obtained for [^{131}I] antibody by the same factor obtained for [^{125}I] normal IgG.

[b] Dissected normal mucosa

[c] Dissected external intestinal wall

therapy of residual cancer cells should be injected in minute amounts in radiolabeled form, first into a nude mouse bearing a human cancer of the same type, then into a cancer patient. Using this method it can be determined by external photoscanning how much of the antibody has the capacity to reach the tumor.

The diagnostic utility of immunoscintigraphy will certainly be increased by advances in nuclear medicine. The single-photon-emission computerized tomography which has been used recently for the detection of our radiolabeled monoclonal anti-CEA antibodies has given very encouraging results (*Mach* et al. 1981; *Berche* et al., to be published). However, there are still some limitations. The spatial resolution was low due to the high energy of [^{131}I]. This isotope should therefore be replaced by [^{125}I] or [^{111}In] if the coupling method suggested gives satisfactory results.

In conclusion, we think that one should be satisfied with the few favorable cases of tumor detection obtained to date by the presently available methods but that more research effort is needed to make the immunoscintigraphic detection of cancer clinically useful.

4 Summary and Conclusions

Monoclonal antibodies (Mabs) against CEA were produced by hybrid cells derived from a fusion between spleen cells of mice immunized with purified CEA and cells from the P3-NSI/1-Ag4 myeloma. The Mabs were purified from hybridoma ascites by ammonium sulfate precipitation and DE-52 chromatography. The purified antibodies which had been shown to have a high specificity and affinity for CEA were labeled with [^{131}I] and injected first into nude mice bearing grafts of human colon carcinoma and later into patients with carcinomas. The results from the clinical studies obtained by external photoscannings and by direct measurement of the radioactivity in resected tumors and adjacent normal tissues showed that monoclonal anti-CEA antibodies can localize specifically in the tumor. Of 28 patients with colorectal or pancreatic carcinoma, 14 gave a positive immunoscintigraph. The specificity of tumor localization was tested by the paired labeling method. A few patients scheduled for tumor resection were injected simultaneously with anti-CEA antibodies labeled with [^{131}I] and normal IgG labeled with [^{125}I]. Differential radioactivity analysis gave specificity indices ranging from 2.2 to 8.4. These encouraging results, however, are not sufficient to recommend the method of immunoscintigraphy for routine tumor detection.

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Automated Systems in Viral Diagnosis

JOHN L. SEVER*

1 Introduction

The age of automation has come to viral diagnosis. A totally automated system is now available for viral antibody determinations and semiautomated methods or components are being used by many laboratories for both antibody and antigen detection. The present paper reviews some of the new methods which are now in use and considers approaches which are under development.

2 Total Automation

A totally automated on-line system for the determination of antibody levels has been available for several years. The system is called the Auto Analyzer II and is produced by Technicon Instruments Corporation, Tarrytown, New York, USA (Fig. 1). It has been marketed primarily for serology tests for the detection of diseases of animals. The system can be converted to perform complement-fixation (CF), hemagglutination (HA), hemagglutination-inhibition (HI), and enzyme-linked immunosorbent assay (ELISA) tests.

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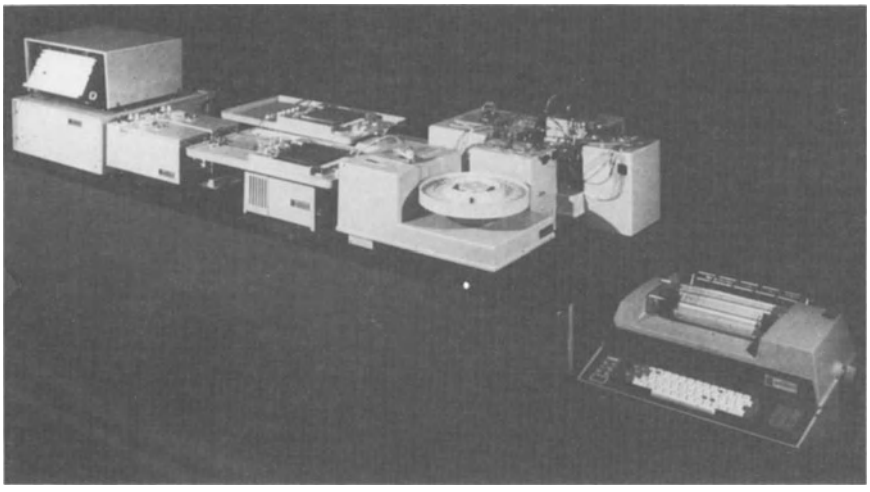

Fig. 1. The Auto Analyzer II, a totally automated system for CF, HA, HI, and ELISA (Technicon Instruments Corporation, Tarrytown, New York, USA)

This system is very similar to the methods marketed by Technicon for a number of years and used by many hospital laboratories for multiple automated blood-chemistry determinations. The continuous-flow method includes (a) a rotary carrousel sampler tray with aspirator for automatic introduction of the samples; (b) a segmented, proportioning air pump with "spaghetti" tubing to carry the samples and add reagents; (c) timed incubation coils; (d) an incubation sampler with multiple reaction tubes and aspirator for longer periods of incubation; (e) two colorimeter readers; and (f) a pen recorder or computerized digital output which is interpreted against reference values. The output can also be routed to a computer for storage and further analysis.

The Technicon system is fully automatic and has the benefits of continuous-flow analysis and microprocessor technology. The approach is particularly applicable to high-volume testing. The digital output can be transferred directly to a central computer for further analysis. The system is marketed for CF, HA, and HI procedures, but it can be converted for ELISA and other tests. The current cost is approximately $ 45 000 for the complete system, and it is estimated that the cost per test is about $ 0.03 including reagents and expendables, and $ 0.15 when labor is included. This, of course, varies with the cost of the antigens and specialized control reagents.

With this method the undiluted sample volume required per test is 10–30 µl and the system processes 30–300 samples per hour. The microprocessor control permits variation in incubation times, reagent, volumes, analysis rates, and sample-to-wash ratios. The bench space required is approximately 1 × 2.5 m.

Although this method was originally developed for large-scale screening of animals presented for slaughter (*Saunders* et al. 1979), it has been used by various laboratories for a number of bacterial and viral diseases (*Downing* et al. 1976; *Meyer* 1979; *Saunders* et al. 1979). This method should be of particular value to laboratories in which large-scale testing is performed, such as certain commercial laboratories or government testing programs. In addition to antibody determinations, it should be possible to adapt the method to viral antigen detection using ELISA or other methods.

3 Partial Automation

A number of methods are available for partial automation of various steps in antibody or antigen detection for viral diagnosis. The equipment used for this automation can be considered in relation to the steps used in most tests.

3.1 Samplers

Several devices for automated sampling are marketed by the Technicon Instruments Corporation. The systems involve placement of the undiluted specimens in cuvettes in blocks or carrousel trays (Fig. 1). The samples are advanced mechanically and an aspirating tube withdraws a predetermined volume of each specimen. The aspirator moves to a wash solution between samples.

3.2 Dispensers

A variety of hand-operated, semiautomated, and fully automated instruments are helpful for dispensing constant volumes of reagents. A hand-operated syringe unit with eight dispensing tips is the Dynadrop SR made by Dynatech Laboratories, Alexandria, Virginia, USA (Fig. 2). This unit delivers drops of 25 or 50 µl. A similar instrument is

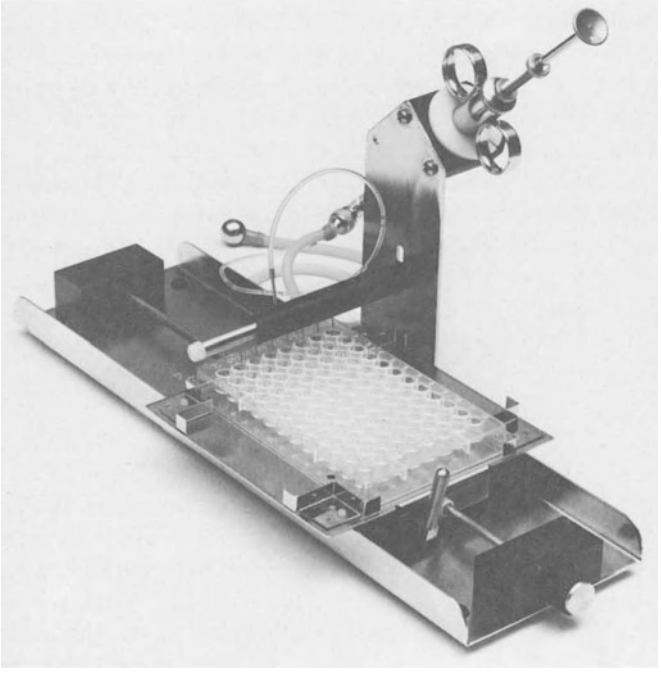

Fig. 2. The Dynadrop SR dispenser, a manually operated dispenser for volumes of 25–50 µl (Dynatech Laboratories, Alexandria, Virginia, USA)

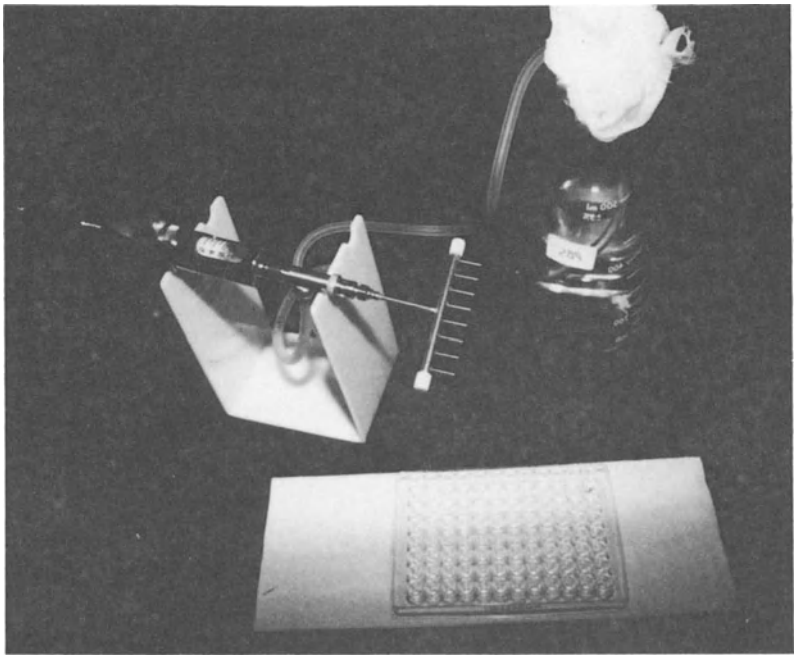

Fig. 3. The hand-operated ELISA Wash Dispenser (M.A. Bioproducts, Walkersville, Maryland, USA)

available for delivering larger volumes of wash fluid for ELISA tests (Fig. 3). This is the ELISA Wash Dispenser made by M.A. Bioproducts, Walkersville, Maryland, USA.

A semiautomated dispenser is the 96-Channel Autopipetter made by Dynatech Laboratories (Fig. 4). This instrument is made for the microtiter system and will pick up and dispense simultaneously 96 aliquots of 25 or 50 µl into a microtiter plate.

Several automated dispensers are available for microtiter plates. The Dynadrop MR (Dynatech Laboratories) pumps constant volumes of the same or different reagents through 12 dispensing tips (Fig. 5). The Titertek Autodrop (EFLAB OY, Helsinki, Finland and Flow Laboratories, McLean, Virginia, USA) is a microprocessor-controlled dispenser with automated volume control and plate advance (Fig. 6). Volumes in the range 10–300 µl can be selected.

3.3 Diluters

Single or serial dilutions can be made in microtiter plates with hand-operated diluters which can be adjusted for volumes in the range 5–50 µl or 50–200 µl (Fig. 7). These units are available in 4-, 8-, and 12-channel configurations (Titertek instruments, EFLAB OY).

A hand-held diluter for microtiter plates called the Rotatiter is available from Dynatech Laboratories (Fig. 8). This unit can hold up to 12 "tulip" loop diluters for serial dilutions. An electric motor rotates the loops when they are placed in successive wells in the plates. Loops are available for volumes of 25 and 50 µl.

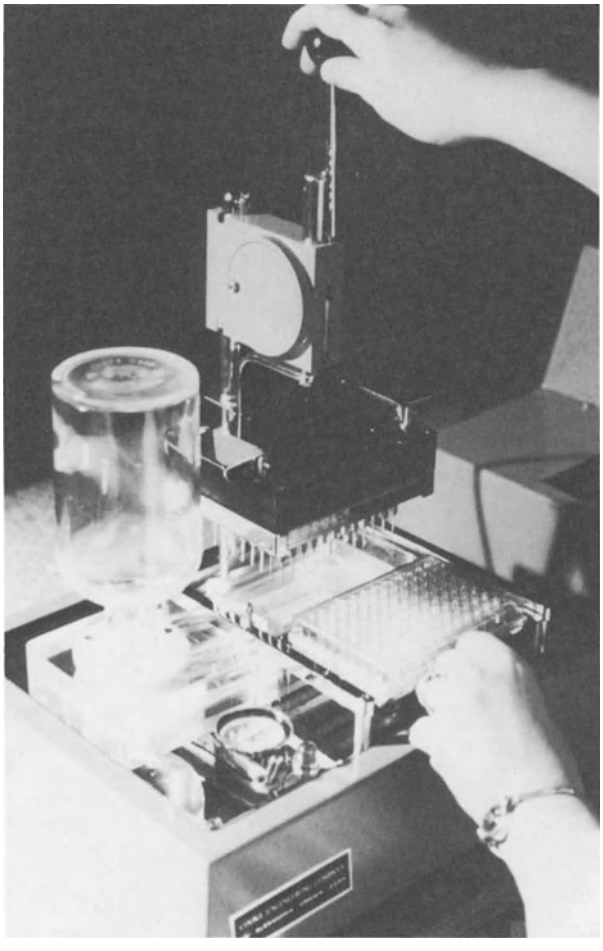

Fig. 4. The 96-Channel Autopipetter a semiautomated dispenser for microtiter volumes of 25 or 50 μl (Dynatech Laboratories, Alexandria, Virginia, USA)

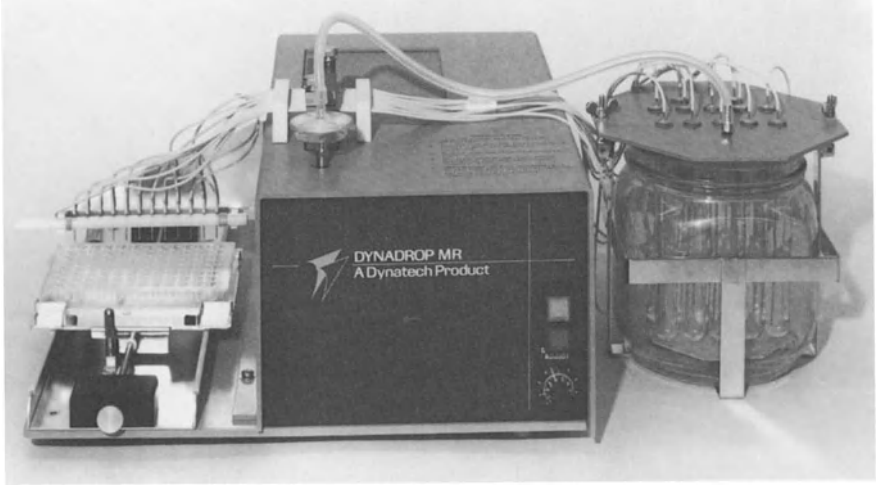

Fig. 5. The Dynadrop MR dispenser, an automated pumping dispenser with 12 tips (Dynatech Laboratories, Alexandria, Virginia, USA)

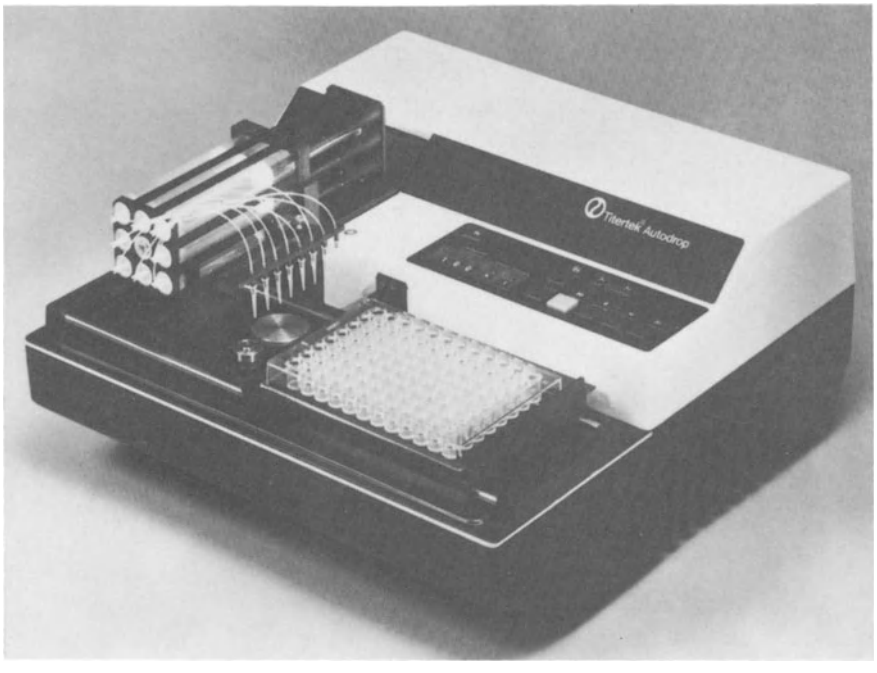

Fig. 6. The Titertek Autodrop, an automated dispenser, microprocessor-controlled, for volumes of 10–300 μl (EFLAB OY, Helsinki, Finland and Flow Laboratories, McLean, Virginia, USA)

An automated diluter, dispenser, aspirator is produced which performs serial dilutions as well as dispensing and aspirating preset volumes in microtiter plates (Fig. 9). This unit can be preprogrammed to perform all three functions in various configurations in the plates (Auto III with SRD III, Dynatech Laboratories). Another automated diluter-dispenser is made for the larger cuvette blocks (Fig. 10). This system is microprocessor-controlled and can make single or serial dilutions (FP-801 Sampler, Labsystems Oy, Helsinki, Finland).

3.4 Transfer Devices

When replicate samples are needed for performing multiple tests on the same samples, transfer devices can be used to withdraw aliquots from a common source or sources. For this purpose we have used the semiautomated 96-Channel Autopipetter for transferring reagents such as complement or diluted red blood cells from a common source, or multiple diluted samples from a microtiter plate to other plates (Fig. 4). Handoperated diluters can also be used for making multiple transfers (Fig. 7).

A new replicate transfer device is now available for simultaneously transferring 96 aliquots of 25 or 50 μl from one microtiter plate to another (Fig. 11). This Replica Plater from Dynatech Laboratories can be autoclaved. It can also be used for transferring samples from hybridoma cultures in microtiter plates.

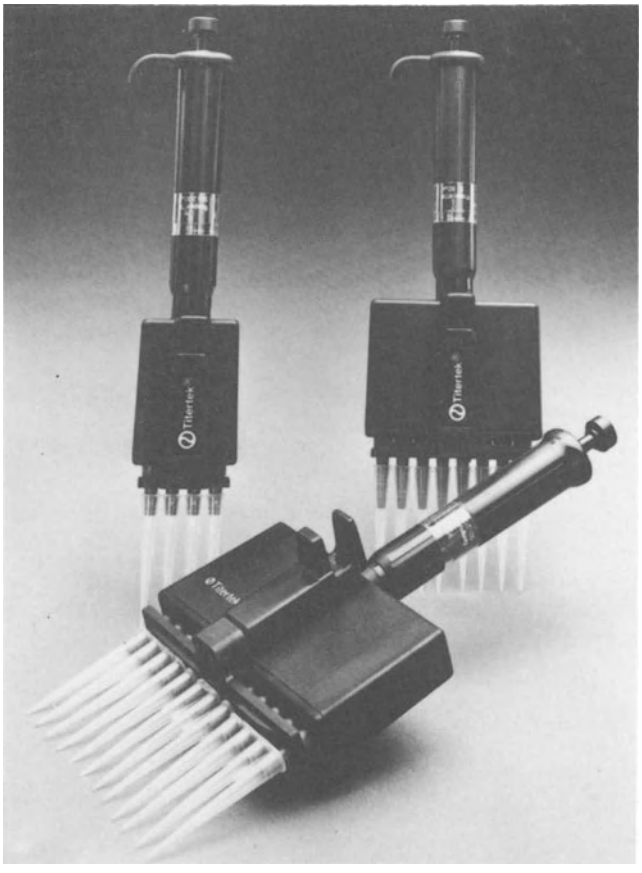

Fig. 7. Titertek hand-operated diluters for 5–50 μl or 50–200 μl volumes (EFLAB OY, Helsinki, Finland)

3.5 Washers

A hand-operated washer-aspirator we have used for microtiter plates is the Miniwash by Dynatech Laboratories (Fig. 12). Plates are advanced by pushing down on the handle, and wash fluid is delivered to the cups by pressing a plunger with the thumb. Individual aspirators connected to a vacuum remove the fluid from the cups as the plate is advanced. This is a particularly useful device for ELISA tests.

A semiautomated washer-aspirator is available, Dynawasher II (Dynatech Laboratories), in which the 96-well microtiter plate is moved to the right for aspiration and to the left for dispensing wash fluid (Fig. 13). The volume of wash fluid dispensed can be controlled.

A fully automatic, programmable washer-aspirator for microtiter plates is also available (Fig. 14). Three different programs for washing and aspirating can be selected; maximum washing time is 1 min (Multiwash, Titertek, Flow Laboratories).

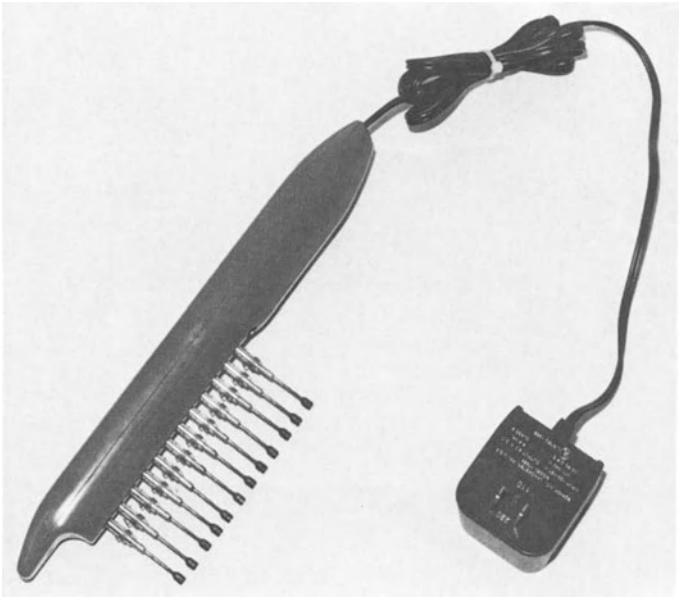

Fig. 8. The Rotatiter, a hand-held diluter for microtiter plates. "tulip" loop diluters are electrically rotated for serial dilutions (Dynatech Laboratories, Alexandria, Virginia, USA)

Fig. 9. The auto III with SRD III, an automated diluter, dispenser, and aspirator for microtiter plates (Dynatech Laboratories, Alexandria, Virginia, USA)

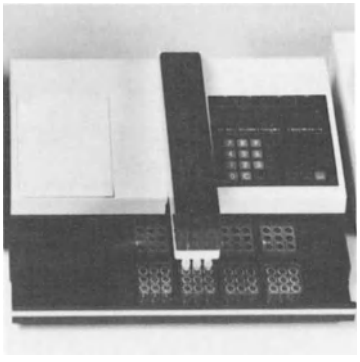

Fig. 10. The FP-801 Sampler, a programable diluter-dispenser for larger cuvette blocks (Labsystems OY, Helsinki, Finland)

3.6 Readers, Printers, and Computerized Analysis

A variety of automated readers are now available for diagnostic virology. Most are designed for ELISA or fluorescent antibody (FA) tests. Various automated beta and gamma counters have been used for radioimmune assays for a number of years. Some readers are linked to printers and computers for data recording, interpretation, and analysis. Almost all ELISA readers provide filters or other methods for changing the wavelengths used by the unit. Some are designed for specific methods marketed in kit form for viral diagnosis. Examples of some of the equipment which is available include:

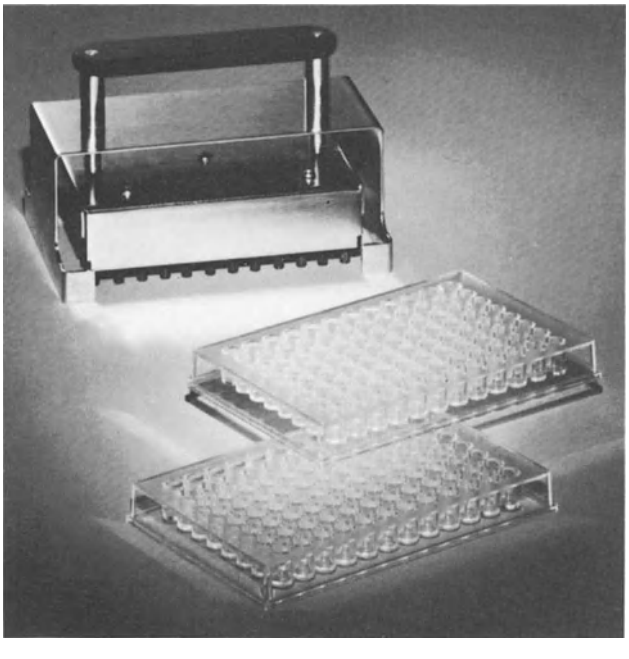

Fig. 11. The Replica plater, a transfer device for simultaneous movement of 96 volumes of 25 or 50 μl from one microtiter plate to another using "tulip" diluters (Dynatech Laboratories, Alexandria, Viriginia, USA)

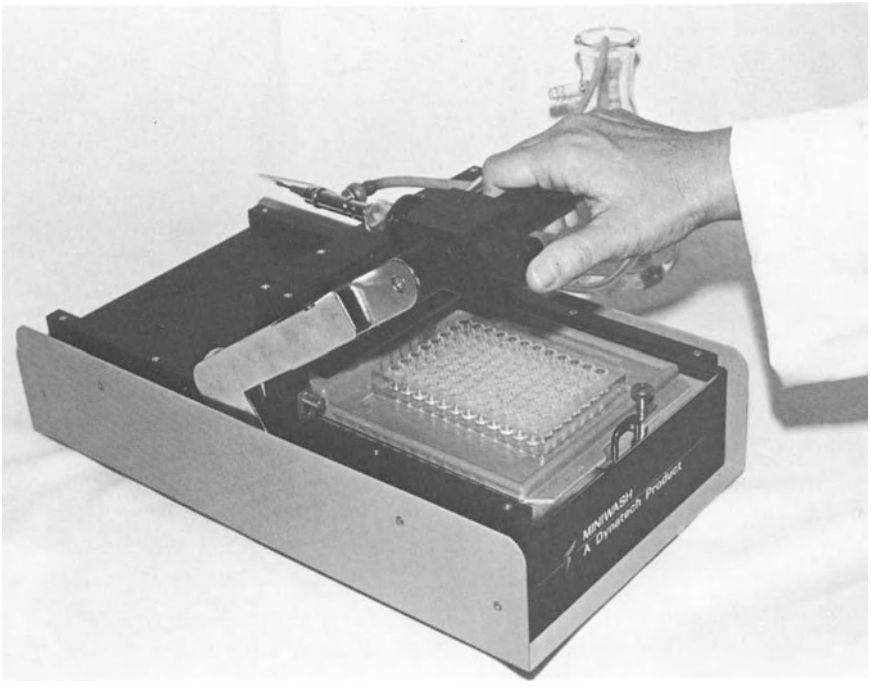

Fig. 12. Miniwash manual washer-aspirator for microtiter plates (Dynatech Laboratories, Alexandria, Virginia, USA)

Fig. 13. The dynawasher II, a semiautomated washer-aspirator for microtiter plates (Dynatech Laboratories, Alexandria, Virginia, USA)

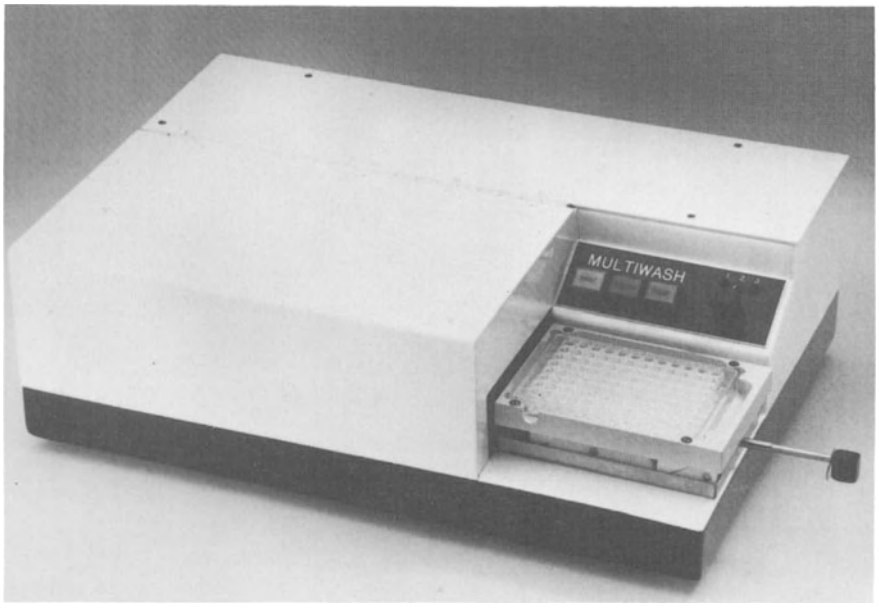

Fig. 14. The Titertek Multi wash, a fully automated, programmable washer-aspirator for microtiter plates (Flow Laboratories, McLean, Virginia, USA)

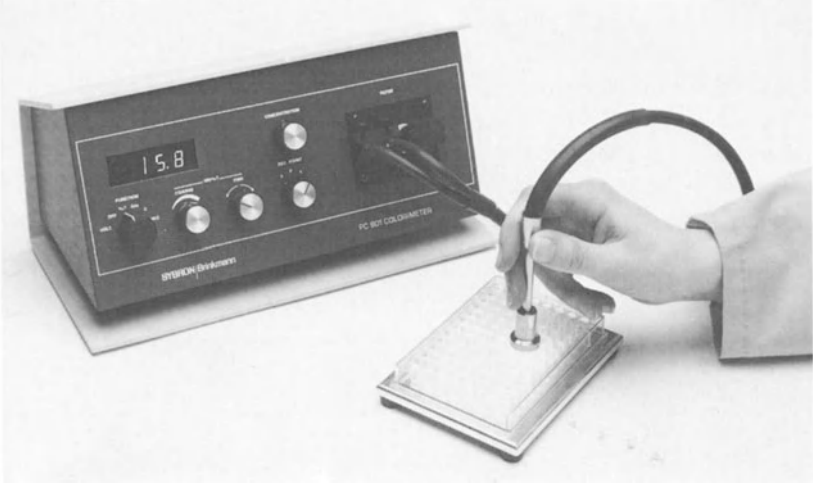

Fig. 15. The Brinkman/Organon Microtiter Plate Reader, a hand-held reader for ELISA using fiber optics (Brinkman Instruments, Westbury, New York, USA)

Brinkman/Organon Microtiter Plate Reader (Brinkman Instruments, Westbury, New York, USA). A hand-held reader with a probe tip using fiber optics (Fig. 15). The tip fits over the microtiter well and light enters the well. The light is reflected back to a photocell and displayed as digital or analog units of absorbance or transmittance.
DU-8 Microplate Analyzer Accessory (Beckman Instruments, Inc. Irvine, California, USA). An attachment to the Beckman DU-8 UV/VIS Computing Spectrophotometer

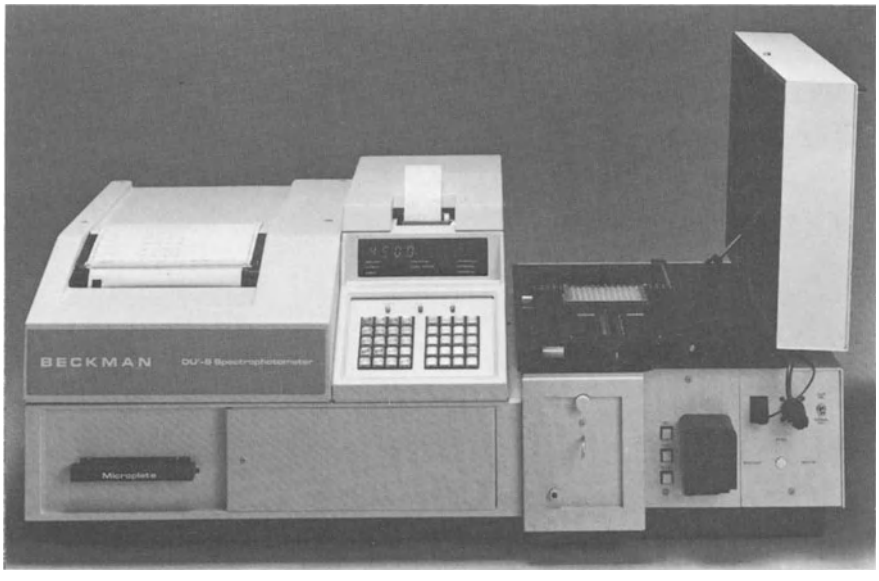

Fig. 16. The DU-8 Microplate Analyzer Accessory, an attachment to the DU-8 UV/VIS Computing Spectrophotometer for ELISA (Beckman Instruments Inc., Irvine, California, USA)

slab gel which permits analysis of microtiter plates (Fig. 16). This unit uses single or dual wavelengths and has a digital plotter.

Gilford Automated EIA System (Gilford Company, Oberlin, Ohio, USA). This reader is designed for the Gilford Cuvette-Paks which come in strips of ten wells (Fig. 17). These cuvettes are also available "precoated" with various "TORCH" antigens for antibody determinations. The Processor-Reader also comes with a peristaltic system for introduction of wash fluid, aspiration, and dispensing of reagents. The system is microprocessor-controlled and a thermal printer is available for recording of test results.

Fig. 17. The Gilford Automated EIA System, for Cuvette-Paks, microprocessor-controlled with a thermal printer (Gilford Company, Oberlin, Ohio, USA)

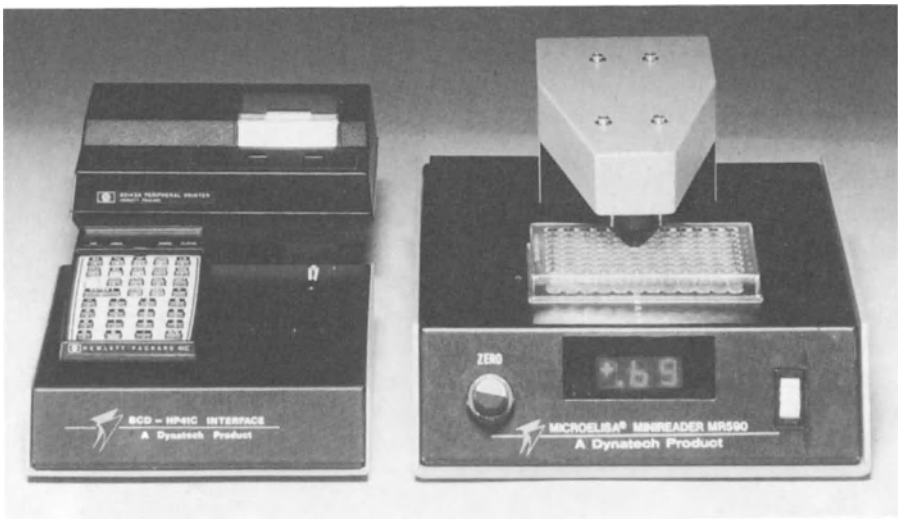

Fig. 18. The MicroELISA Mini Reader, a manually operated, less expensive reader which can be linked to a calculator-printer (Dynatech Laboratories, Alexandria, Virginia USA)

MicroELISA MiniReader MR 590 (Dynatech Laboratories). This is a small, relatively inexpensive photometer for "U"- or flat-bottom microtiter plates (Fig. 18). The plate is moved manually under a cone which centers the beam and gives a digital display of absorbance. The unit can be linked to a calculator interface for data analysis and printing of results.

EIA Reader Model 307 (Bio-Tek Instruments Inc., Burlington, Vermont, USA) with this reader microtiter plates are advanced manually and the reading head engages each

Fig. 19. EIA Reader Model 307. Plates are manually advanced and LED displays indicate optical density and plate position. An interface and printer are available (Bio-Tek Instruments Inc., Burlington, Vermont, USA)

well (Fig. 19). Optical density is shown on an LED display, along with plate position. An interface and printer are available for this unit.

Microplate Reader MR 600 and Apple Computer System (Dynatech Laboratories). This unit has a dual wavelength system for measuring absorbance through microELISA or microtiter plates (Fig. 20). It automatically positions and advances the plate. There is a four-digit LED display of results and a printer. This system reads a 96-well plate in approximately 1 min and can be interfaced with an Apple computer for data storage and analysis.

TiterTek Multiskan (EFLAB OY and Flow Laboratories). A vertical through-the-plate reader which measures eight wells at a time and automatically advances the microtiter plate (Fig. 21). This unit prints results as absorbance or "matrix" and reads a 96-well plate in 1 min. An interface is available so that the unit can be connected to a computer. A similar model FP-901 analysis is also available for use with the larger nine-well cuvettes shown with the diluter-dispenser (Fig. 10).

Artek Model 210 Reader and Computer (Artek Systems Corporation, Farmingdale, New York, USA). This computer-controlled reader automatically positions and reads the 96-well microtiter plate in 45 s (Fig. 22). There is a video display and printout. Up to 52 800 readings can be stored on disks and analyzed in the computer. The user can program the computer, or customized programing is available.

Bionetics Autoreader LBI-321 (Litton Bionetics, Kensington, Maryland, USA). A programmable reader which scans eight microtiter wells at a time and automatically advances the plate (Fig. 23). It reads at a single wavelength (690 nm). An attached computer will analyze and print results. The instrument can be set for "screen" or "titer" modes for 1–4 antigens. It is designed to work with the Bio-Enza-Bead system marketed by Litton Bionetics for antibody to the TORCH reagents and other antibody

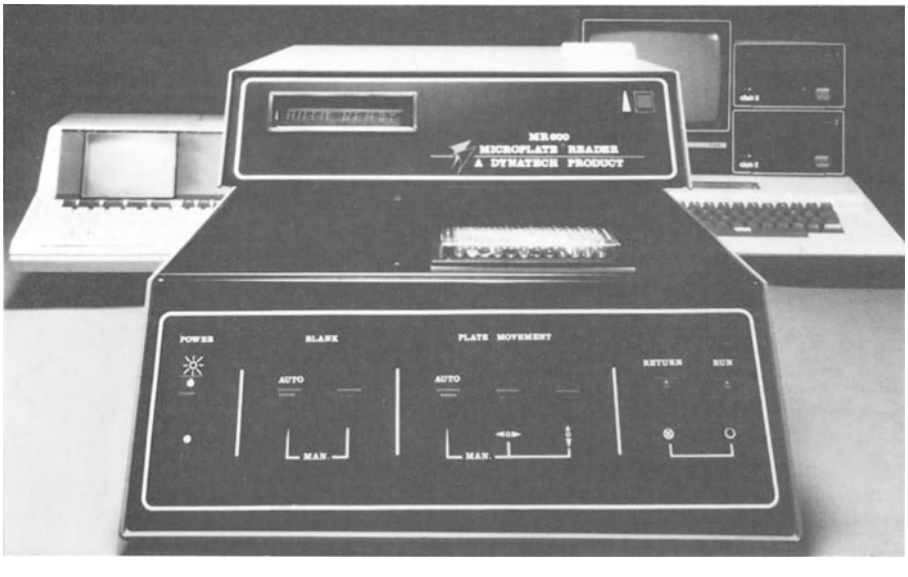

Fig. 20. Microplate Reader MR 600, a dual wavelength, automatic advance reader which can display and print results and can be linked with an Apple computer for data analysis (Dynatech Laboratories, Alexandria, Virginia, USA)

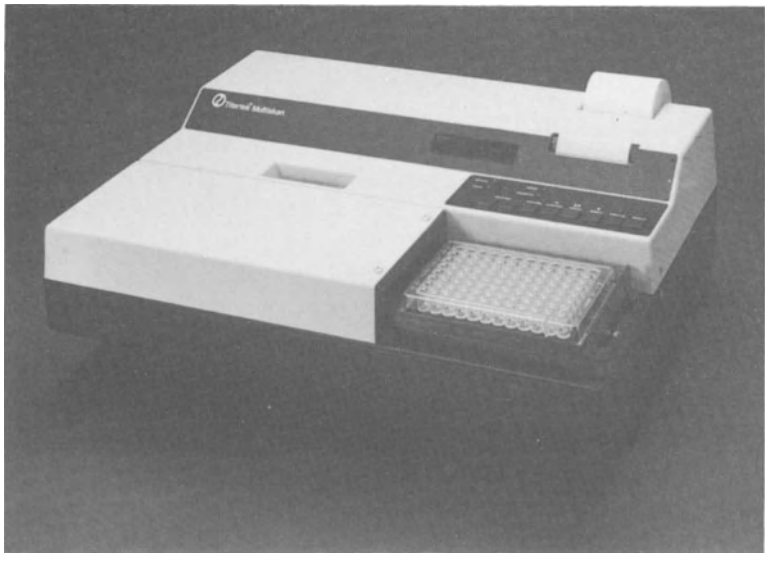

Fig. 21. The Titertek Multiskan, a unit that reads 8 wells simultaneously and automatically advances the plate, prints the results, and an interface is available for connection to a computer (EFLAB OY, Helsinki, Finland and Flow Laboratories, McLean, Virginia, USA)

systems. With this system, beads are introduced individually and can be washed automatically in a new magnetic transfer device.

Quantum I (Abbott Laboratories Diagnostics Division, North Chicago, Illinois, USA). A preprogrammed dual-beam reader for single-tube ELISA tests (Fig. 24). The protocols for tests are stored and printed. The instrument compares absorbance with controls and plots values as well as percent activity. The program can be modified. The unit reads one tube per second, is microprocessor controlled, and flags abnormal

Fig. 22. The Artek Model 210 Reader and Computer, a computer-controlled reader with video display and printout. Data can be stored on disks and analyzed (Artek Systems Corporations, Farmingdale, New York, USA)

Fig. 23. The Bionetics Autoreader LBI-321, a programmable reader which scans eight microtiter wells simultaneously, and computer analyzes and prints the results; designed for the Bio-Enza Bead system. An automatic magnetic transfer device is also available (Litton Bionetics, Kensington, Maryland, USA)

Fig. 24. Quantum I, a preprogrammed reader for single-tube ELISA tests which plots values and gives percent activity (Abbott Laboratories, Diagnostics Division, North Chicago, Illinois, USA)

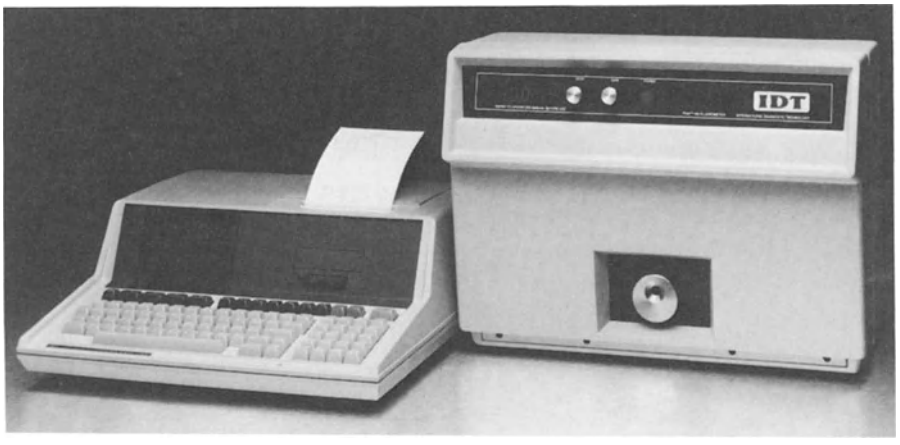

Fig. 25. The FIAX 100 Digital Florometer, a fluorometer for flat sticks (StiQ) with test-dedicated cassette in computer; designed for rubella and other antibody determinations marketed by IDT (International Diagnostic Technology (IDT), Santa Clara, California, USA)

results. The system was developed for ELISA assays for hepatitis antigens and antibody as well as rotavirus antigen, rubella antibody, and other systems marketed by Abbott Laboratories.

FIAX 100 Digital Fluorometer (International Diagnostic Technology (IDT), Santa Clara, California, USA). A digital fluorometer in which samples on flat sticks (StiQ) are introduced into the central hole (Fig. 25). The instrument is microprocessor controlled, and fluorescence is measured, displayed, and printed. Data can be processed in a compatible computer. This unit is produced to work with the rubella StiQ produced by IDT and with other viral antibody systems.

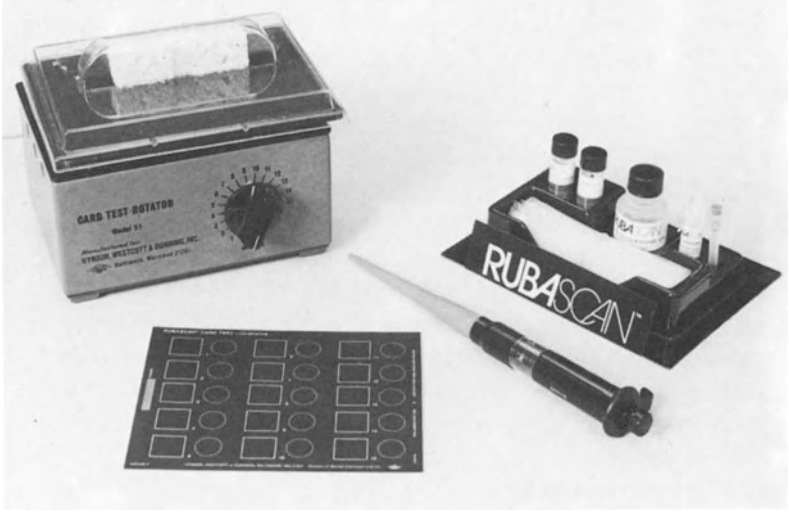

Fig. 26. Rubascan latex agglutination test, a 10-min card-agglutination test for rubella antibody. This rapid latex test avoids many of the steps required by other methods and can be performed easily in a physician's office, clinic, laboratory, or in the field (Hynson, Wescott and Dunning, Baltimore, Maryland, USA)

4 Latex Agglutination

A new 10-min latex agglutination test, Rubascan, is now available for rubella antibody screening (Hynson, Wescott and Dunning, Baltimore, Maryland, USA). With this method 25 μl serum is mixed with 15 μl rubella-sensitized latex on a card and rotated for 8 min under a humidifying cover (Fig. 26). The card is then read visually for agglutination of the white latex. The serum can also be diluted to determine the titer. This method avoids many of the steps required by the other procedures. It can be used in a physician's office, clinic, laboratory, or in the field. Since the results are available in a few minutes, the physician can immediately counsel the patient and immunize, if necessary. While the simplicity of the present test avoids the need for automation, if large numbers of sera were to be run, automation of sampling, mixing, and reading would be desirable. The method should be applicable to other virus antibody and antigen tests.

5 Summary and Conclusions

The automated systems available for viral diagnosis are approaching the standard of those which have been marketed for a number of years for clinical chemical tests. The totally automated Technicon instrument was developed for large-volume use. To date, it has been utilized primarily in veterinary viral and bacterial diagnostic centers. With large-volume testing now being conducted for human viral infections such as hepatitis and rubella, this method should be of value to the larger commercial and government laboratories.

Semiautomated systems or components have been developed for almost every step used in rapid viral diagnostic procedures. Much of the equipment has been made for the microtiter system using 96-well plates (*Sever* 1962). A large number of premade kits have also been produced for TORCH and other reagents (*Castellano* et al. 1981; *Sever* and *Cleghorn* 1982). Many of these use the microtiter system and the new related equipment for semiautomation. Some manufacturers produce a "line" of semiautomated components which, when placed next to each other, provide an almost completely automated system. A few manufacturers have produced instruments specifically for their test kit systems in order to automate the processing of specimens.

Clearly, the purpose of automation is to reduce time and costs for the performance of tests. Savings can also be achieved by keeping the volumes of reagents low and improving precision by using equipment to perform repetitive tasks and precise readings. Simplification of methods such as that which has been achieved with latex agglutination avoids many time-consuming steps. Manufacturers are producing the new equipment needed for modern rapid diagnostic methods in virology. There is an obvious movement toward total automation, and the future should bring us more automated systems for our laboratories.

Acknowledgments. We are grateful for the cooperation of the manufacturers for supplying photographs and specifications for their instruments. Because of the large variety of equipment available, it was not possible to include all manufacturers and all instruments.

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Data Processing in Clinical Virology

KARL-OTTO HABERMEHL*

1 Introduction

Laboratory diagnosis of viral diseases requires an individually defined course of highly specialized laboratory procedures. In order to obtain relevant results it is necessary to get detailed data concerning the patient as well as clinical information concerning the type of disease and the date of its onset, including any special symptoms which may be present. On the basis of this information, the diagnostician in the laboratory has to decide what type of tests are to be performed and, depending on the preliminary results, which additional diagnostic procedures are necessary. Finally, the results of the laboratory tests together with the clinical symptomatology and the epidemiological or endemiological situation enable a report to be compiled.

The accumulation of large numbers of samples to be examined by different methods on the one hand, and the necessity to decide on a specific test for an individual patient on the other, is a contradiction in itself. This problem can be solved only by electronic data processing. Moreover, this information should be available for im-

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mediate access over a long period of time in case of later requests. In order to fulfill these requirements, it is necessary to computerize the available diagnostic data (*Vroman* 1979).

2 Concept for a Laboratory Computer for Clinical Virology

From the numerous proposals concerning the requirements for computer systems in diagnostic work in clinical microbiology (*Krieg* et al. 1971; *Kobernick* and *Mandell* 1974; *Ridgway* et al. 1980; *Williams* et al. 1978), it is now generally agreed that laboratory computers should be both easy to operate and accessible to laboratory personnel (*Walter* 1973). The programs should be designed in such a way that they can be adapted by laboratory personnel to specific needs without the assistance of a programmer. In addition, all data files should be available for immediate access. If diagnostic work is to be performed on a larger scale, even relatively short delays in the response to inquiries render the system impractical.

The combined requirements of immediate access and long-term storage of data lead necessarily to the application of decentralized minicomputers. Moreover, it has been suggested that the laboratory computer should be an independent unit under the supervision of the laboratory (*Kobernick* and *Mandell* 1974; *Rappaport* et al. 1969). Based on these considerations and on the experiences of other authors (*Blair* and *Brown* 1981; *Farrar* et al. 1975; *Goodwin* 1976; *Goodwin* and *Smith* 1976; *Harvey* et al. 1972; *Mitchison* et al. 1978; *Vermeulen* et al. 1974), we developed a concept for a laboratory computer for clinical virology (*Habermehl* 1980, 1981, to be published) that allows institute personnel to handle the system without any special knowledge or training.

Using a dialogue system, data input is performed in clear text or as abbreviations of the clear text of variable length, starting with the initial letters of selected words. The use of code numbers is avoided as much as possible. All data are stored for as long as possible, at least for several years. They remain available for immediate access and for linkage with the results from new diagnostic investigations. Thereafter, the data from previous tests are stored on tape, ensuring that the name and date of birth of the patient remain on the disc together with a reference to the location of his personal data file.

According to the individual request, working instructions are printed for specific diagnostic procedures, referring to the titers or results from previous tests of the patient, in order to facilitate diagnostic assays. After input of the test results, the system prints an interim diagnostic report for laboratory use only, containing all clinical data and results including linkage analysis, diagnosis, and information on previous requests. This interim report, printed out in mnemonics, enables the virologist to give a formatted interpretation of the results or a virological diagnosis, or to make additional remarks is necessary.

Upon request of the laboratory, the system gives a detailed report to the consulting doctor or a ward report in clear text without mnemonics, containing all data (present and previous) and laboratory results concerning the patient. The conclusion of this report is a virological diagnosis, and if necessary the report should contain additional information. Interim reports are also possible.

Programs for statistical procedures are also included in the dialogue system. The linkage system allows for detailed statistical analyses of all data with any desired logical connection, for example, establishment of a serum bank with listings of the different antibody titers against several antigens. Further information such as an alphabetical listing of patients or data files is also available. In addition, the system performs all administrative work including the complete statement of accounts.

3 Functions of the Computer System

In order to obtain a comprehensive survey of the problem of data processing in clinical virology, it is necessary to demonstrate all steps of the diagnostic procedure.

3.1 Hardware

The system was started in 1975 as a pilot project using a small computer, the Wang 2200C: memory, 16 kilobytes; disc, fixed/removable, 4 × 5.5 megabytes; printer, 180 characters/s. After a thorough revision, the system was established in a larger computer, the Digital PDP L/34: multi-user operating system; memory, 256 kilobytes; disc, removable, 2 × 30 megabytes; printer, 300 lines/min. The software was developed by Dr. Buder Computer GmbH, Berlin.

3.2 Input of Clinical Data into the System

The software is designed for manual entry of information using terminals. It would be desirable to enter the data on prepunched or mark-sense cards, but this requires accurate and time-consuming work by the admitting service. Due to the variety of senders (several hospitals, general practitioners, pathologists, etc.) we have found that only the manual entry of information is practicable.

In order to obtain sufficient information in printed form which is easy to survey, we developed a single-sheet requisition form containing space for personal data of the patient and a listing of a selection of symptoms or syndromes. Three different symptoms can be marked by the sender. The type of test procedure can be proposed by the sender in clear text, but in the case of virological work it is advisable that the laboratory makes the final decision on the assay methods.

Patient admittance is the crucial problem of any computer system in clinical virology. We therefore developed time-saving procedures for the input of data concerning patients, senders, clinical diagnosis, and required investigations. Since most of the submitted samples are received from a limited number of senders, a data file exists containing their full addresses and any other pertinent information. For the admittance of a patient, it is necessary to answer the question concerning the identity of the sender with only the first two (or more) letters of the name. For example, the input of "ST" will be followed by the response "STEGLITZ KLINIKUM", and if the response is confirmed by the operator, the input of the complete information is fixed. If the sender's name is not stored in the data file, the input has to be performed immediately using a simple procedure regulated by the dialogue system. This time-saving principle

has also been established for the input of clinical diagnoses, assay methods, specimen and virus designations, etc. The time-saving system of input, i.e., working with the initial letters of special terms instead of using code numbers, does not apply only to the admittance of patients but also to all other computerized procedures, including statistics and administration.

4 Input of Data and of Requests for Testing

The first step in this procedure is shown in Fig. 1. After input of the date of birth and the first two letters of the name of the patient, the system checks within several tenths of a second whether the patient's name is already stored in the file. If the patient's name is filed, the system indicates the number and date of the last admission, followed by the question "INFO" (Is more information desired?). If this is responded to with a "J" (yes), all previous data are presented immediately. Figure 2 illustrates the information called up onto the screen of the terminal, showing the data for the last admission, the number of the sample, personal data of the patient and clinical diagnoses, including the duration of the illness (2 days in this example). The results of the tests are given in mnemonics (only for internal use), followed by the previous virological diagnoses and the earlier information supplied by the institute. After the question: "Function (B/A)" (B, before; A, after) one can decide whether previous admissions

Date of birth	12. 09. 1953
Beginning of surname	MU
Name	Mueller Carola 12.09.53
Last entry	First entry of 05.04.81
Info	No
Pregnant	No
Sender	Steglitz Clinic
Ward	Ward 06
Insurance company	AOK
1 clin. diagnosis	Encephalitis
2 clin. diagnosis	Parotitis
3 clin. diagnosis	Bronchitis
Duration of illness	4
Investigation	5: Viruses causing diseases of the CNS
	7: Viruses causing diseases of the respiratory tract
Specimen	Venous blood
Method	Complement fixing reaction
Virus	Psittacosis
Subgroup	
Virus	
Method	
Specimen	

Correction (J/N/P/X)

Fig. 1. Admittance of patients into the computer. Progress of the input dialogue on the screen of the terminal

03.01.82	01091101/01	Schulz Heinz	M	01.08.52	2 days
Steglitz clinic					
Ward 03		Pneumonia			
VBL-CFT-INA	4/1	VBL-CFT-INB	4/1	VBL-CFT-ADE	8/1
VBL-CFT-PAR-1	4/1	VBL-CFT-PAR-2	4/1	VBL-CFT-PAR-3	4/1
VBL-CFT-RES	4/1				
RAC-TCI:	INA-H3N2				
		Fresh infection with influenza A. Send a sample of venous blood please, in 8–14 days at the earliest			

Function (B/A)

Fig. 2. Information system on the screen of the terminal providing all data concerning a patient form one admission. Information from previous or following admissions can be requested by typing in the letter "B" or "A" (before or after), respectively, to the question "FUNCTION (B/A)"

should be recalled. If in Figure 1 the question "INFO" is answered with "N" (no) the dialogue system requests the name of the sender. As mentioned above, it is sufficient to type in the first letters of the name (for example "ST", and if "ST" is on file, the answer will be "STEGLITZ KLINIKUM"). This same time-saving principle is used for input of the "STATION" (ward number, the "KLIN. DIAGNOSE" (clinical diagnosis), and so on.

Two different modes exist to request the designated laboratory tests. In the single mode the dialogue system asks for the type of specimen ("UNTERSUCHUNGSMATERIAL"), the method of testing ("UNTERSUCHUNGSMETHODE"), and the type of agent ("VIRUS"). Again, it is sufficient to type only the first two (or more) letters, which are then followed by confirmation of the whole term. Thereafter the system continues questioning for "VIRUS", "UNTERSUCHUNGSMETHODE" (method of testing), and "UNTERSUCHUNGSMATERIAL" (specimen). If these last questions are not responded to, the system finishes the input procedure, gives the sample number for this patient, and prints it in sequential numbering order. Thereafter the computer begins the admission program for the next patient.

In the other mode of input, blocks of assays can be requested using code numbers. In this case the input mode for "UNTERSUCHUNGSGRUPPEN" (groups of tests) has to be responded to using a code number. Input of "5", for example, is answered with "VIREN V. ERKR. DES ZNS" (viruses causing diseases of the central nervous system). Input of "7" is answered with "VIREN V. ERKR. DES RESPIRATIONSTRAKTES" (viruses causing respiratory tract infections). This latter group includes the information: complement fixation tests with venous blood for influenza virus types A and B, parainfluenza virus types and 1, 2, 3, respiratory syncytial virus, adeno viruses, and mycoplasm. Several groups of tests can be requested and combined in this mode. Apart from these group requests, it is possible to continue requests in the individual single mode system as described above. This time-saving principle allows for the admission of several hundred individual patients within a surprisingly short period of time. Further time reductions can be obtained by performing the input on several terminals simultaneously.

5 Working Instructions for Different Diagnostic Procedures

Upon request, different working instructions are printed out for all working groups in the laboratory. The worksheets have different patterns according to the type of test to be performed. They remain as working protocols at the different work places in the laboratory. Fig. 3 shows a working protocol for isolations from tissue cultures. Both the sample number and the name of the patient are always printed out simultaneously, in order to avoid confusion. The technician works with this form, recording the different types of tissue cultures, the dates of microscopic examinations, and the results. Another type of protocol is shown in Fig. 4, concerning hemagglutination-inhibition tests with rubella virus. At the top of the sheet the number of the type of protocol is given (in this example "0030"), followed by the identification of the protocol form. The number is necessary in order to retrieve the appropriate program for quick input of results. If previous data exist in the file concerning the same patient, the old sample numbers are listed together with the results. Samples and antigens are marked by the respective mnemonic terms, e.g. "VBL" (venous blood) or "ROE" (rubella). The requested examinations are marked with dotted lines. Listing the previous results is not only time-saving, since it enables the technician to start with the appropriate dilution, but it is also helpful for internal quality control, sometimes indicating unexpected developments in the antibody titer. The same principle is established in some of the other working protocols for serological examinations like complement fixation tests (Fig. 5), hepatitis radioimmunoassays (Fig. 6), or picornavirus-neutralisation tests (Fig. 7). The requested assay procedures are marked with dotted lines.

Apart from the protocol sheets mentioned above an additional *master working protocol* is printed containing all the information and all tests requested for the different work stations. This enables each technician to obtain general information on all the diagnostic steps to be performed with the same sample at different work stations in the laboratory.

6 Generation and Modification of Working Instructions

The application of new methods or the introduction of new antigens in serology requires generation of new working protocols or modification of the old ones. For this reason special programs exist, operating in the dialogue mode, which enable the user to perform generation or modification of protocol forms without special knowledge of programming. To establish a new serology protocol, for example, the system proposes the number of the new form, asks for the name of the form, the type of sample (e.g., venous blood), and the different antigens (up to 20 in one protocol). After these questions have been responded to, the new form is automatically generated in the file, similar to those shown in Fig. 5. In addition, a special program exists which is designed for changing previously established working protocols.

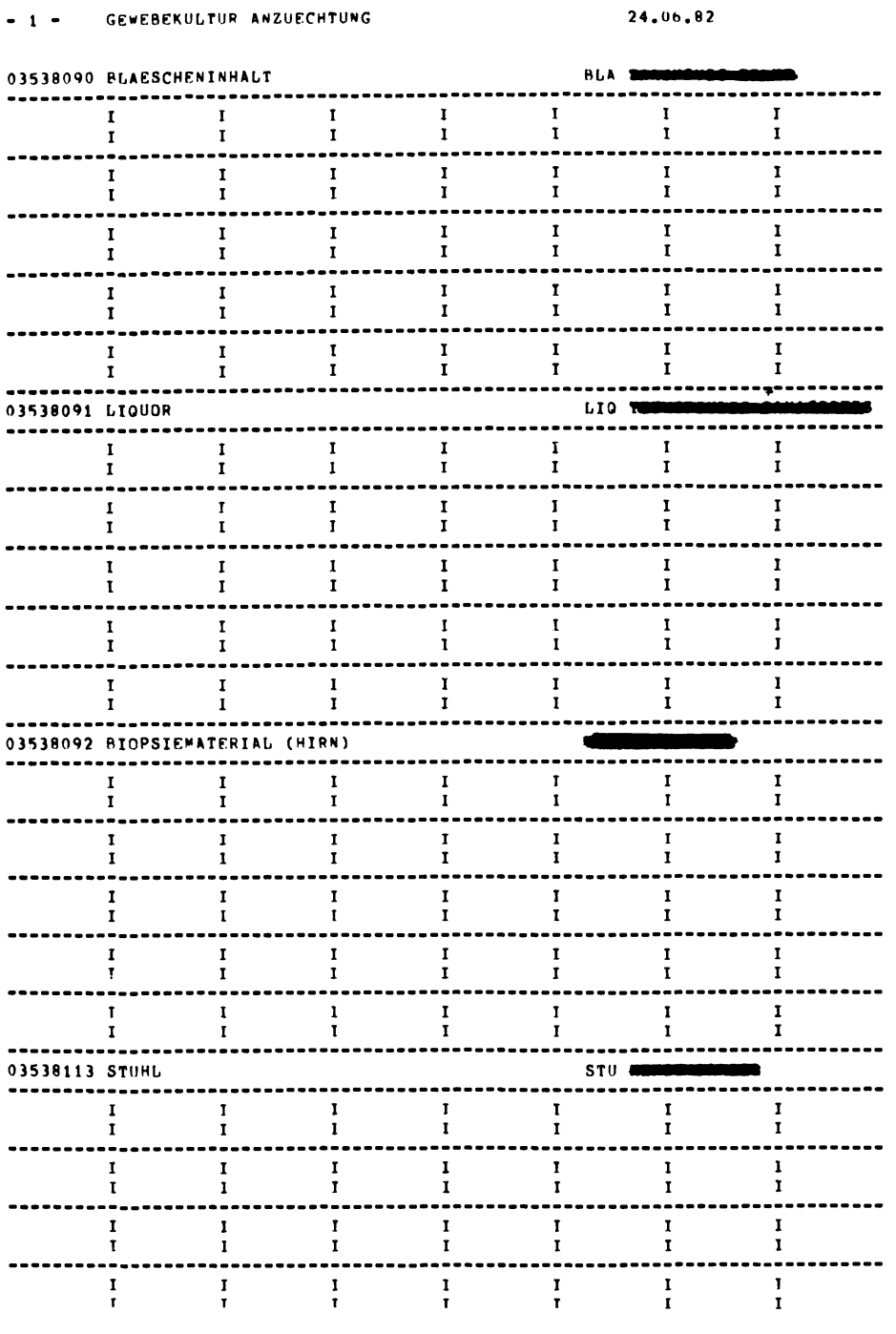

Fig. 3. Printout of a protocol for virus isolation from tissue culture

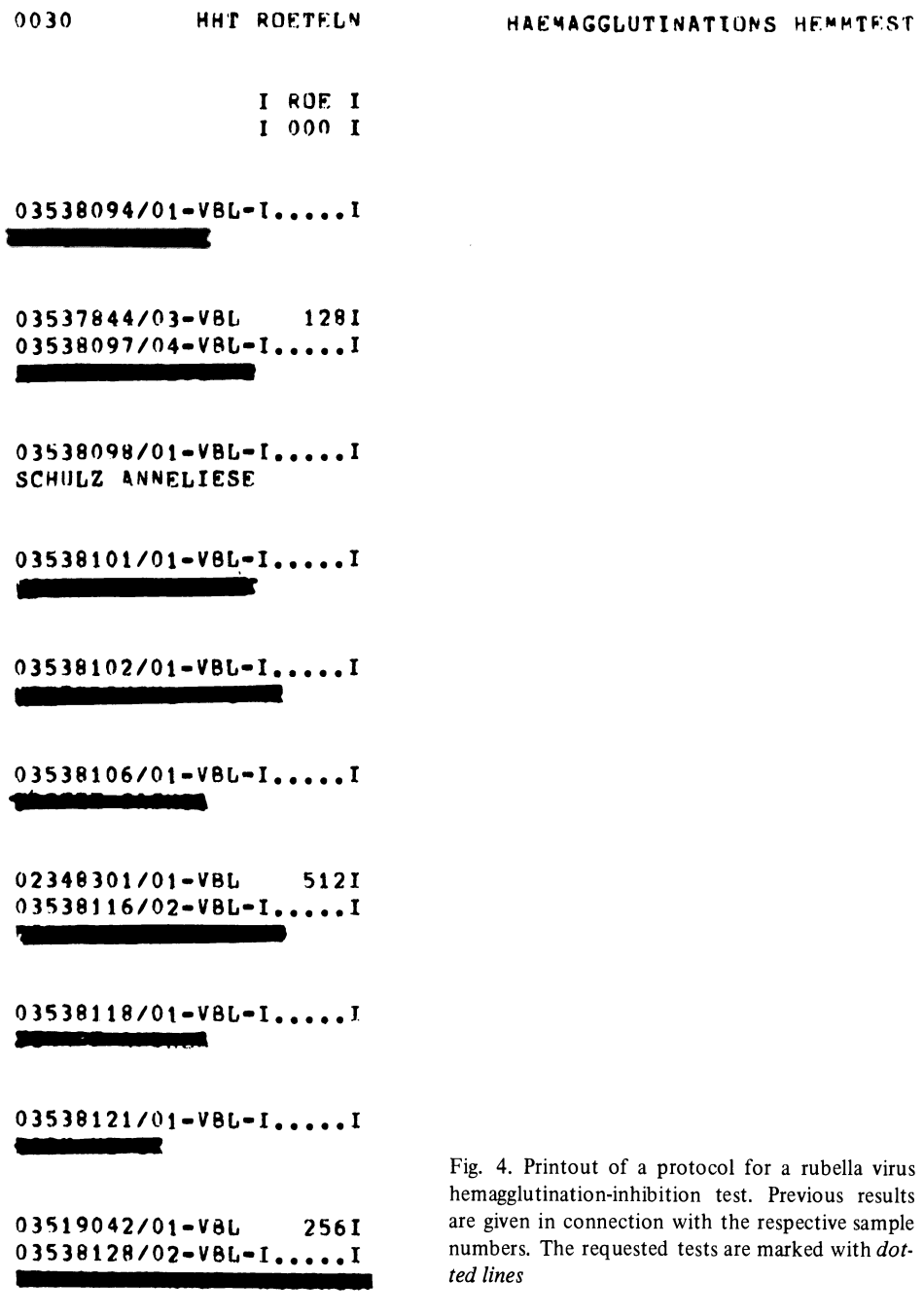

Fig. 4. Printout of a protocol for a rubella virus hemagglutination-inhibition test. Previous results are given in connection with the respective sample numbers. The requested tests are marked with *dotted lines*

Fig. 6. Printout of a protocol for hepatitis radioimmunoassays. The results of previous admissions ▸ are given by the counts of the gamma counter as well as by the significance, positive (+) or negative (–)

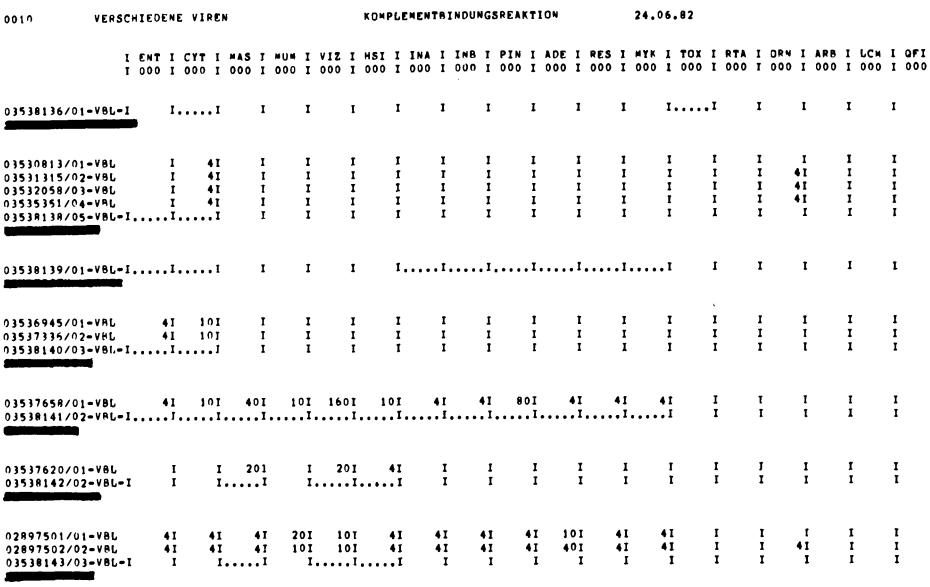

Fig. 5. Printout of a protocol for complement-fixation tests with 18 different antigens marked by mnemonics. Format described in legend to Fig. 4

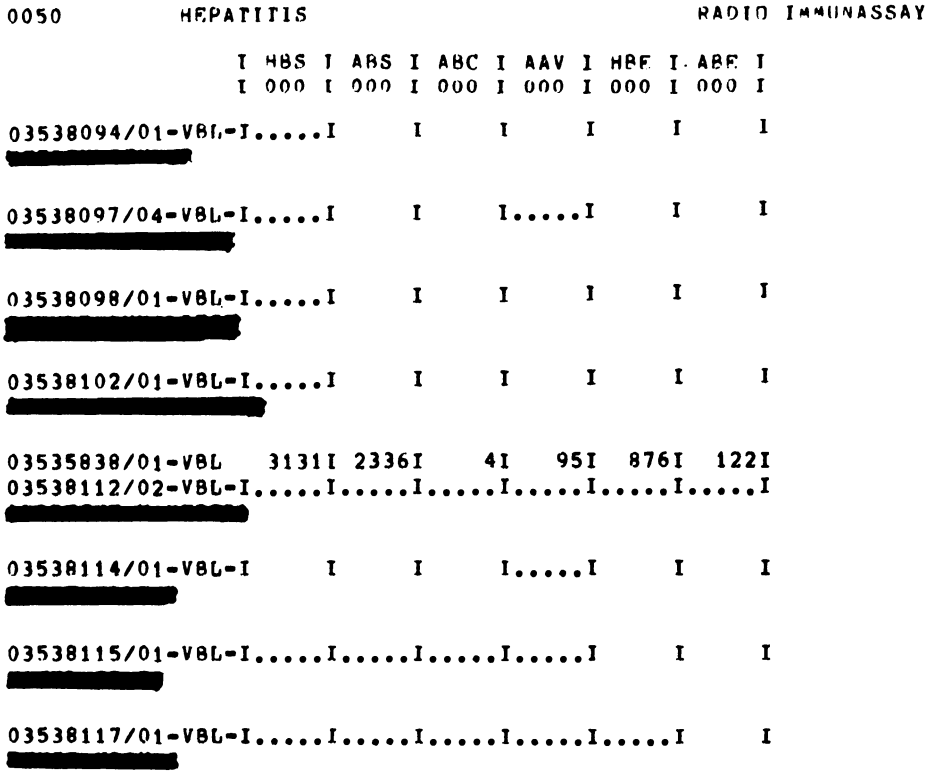

Fig. 7. Printout of a protocol for several picornavirus-neutralisation tests. Format described in legend to Fig. 4

7 Input of Test Results

The system offers two modes for the input of results. In the mode concerning most of the information, the technician requests at the terminal the file for input of results and types in the number of the worksheet. The computer responds by listing on the screen the patient's name and the different tests carried out, in the same order as on the worksheet. The system stops automatically at the space designated for the input of the result. Thereafter, the technician has only to type in the antibody titer and after pressing a key the next space for the input of results appears automatically. The system has proven capable of allowing rapid input of results. It is easier and safer than marking the results on sense cards.

The second input mode for results is based on a program starting after the input of the sample number of the patient and listing the different tests in sequence. This mode permits input and correction of single results from individual patients. Both modes of input offer the possibility for correction at any desired time.

8 Interim Diagnostic Surveys

Upon request, usually once a day, the system prints a diagnostic survey, a "DIAGNOSE LISTE" (list of diagnoses). This is a listing of all patients from whom test results have been obtained since the previous printout of a diagnostic survey. As shown in Fig. 8, this listing consists of a condensed survey of all information on the patients, including results, diagnoses, and a reference to previous admissions. This listing not only allows a survey on the course of the illness, which is the basis for a diagnosis, but it also enables the diagnostician in the laboratory to control the work routine and to request additional measures.

Most of the diagnoses and additional remarks made by the laboratory diagnostician are formatted and stored in a data file. For example, remark "24" means: "PLEASE SEND ANOTHER SAMPLE OF . . . WITHIN THE NEXT 8–14 DAYS." It is only necessary to type the number of the diagnosis or remark in the margin of the listing. These numbers will be introduced into the system in a specially shortened program run by a typist. This is the basis for the medical report to the sender.

Apart from the form text, free texts can also be generated. However, in order to make the diagnoses and additional remarks available for statistical evaluation, form texts are preferred. The data file contains approximately 100 form texts. In the same mode as mentioned above the text can be changed or new texts can be added into the dialogue system without the help of a programmer. In this respect, it should always be kept in mind that adjusting an old text should not change the meaning altogether, since otherwise the statistical evaluation of old and new texts would produce different results. Therefore, rather than adjusting old texts, new texts should be added to the data file.

One becomes accustomed to reading the diagnostic survey list very quickly. The data on more than 100 patients can be scanned in approximately 20 min. This survey is, in this author's opinion, the most important part of the system, as it fulfills the requirements mentioned in the introduction: to combine and link large numbers of test results and data with individual diagnostic procedures in virology.

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***  D I A G N O S E L I S T E  VOM 24.06.82 ***

[redacted] WILLY                                    GESCHLECHT: M GEBR.:  8.07.12

PSYCHIATR.U.NEUROLOG. KLINIK          STATION [redacted]

EINS-DATUM:     I 24.05.82 I
MAT-NR:         I  3536594 I
EINS-NR:        I     1.   I

PARESEN (UNKLA I     *     I

LIQ             I     *     I
VBL             I     *     I

LIQ-GAZ         I  NEGATIV I
VBL-KBR-CYT-000I     10/1  I
VBL-KBR-ENT-000I      4/1  I
VBL-KBR-HSI-000I     20/1  I
VBL-KBR-MAS-000I      4/1  I
VBL-KBR-MUM-000I      5/1  I
VBL-KBR-VIZ-000I      4/1  I

24.05.82  3536594/01
ERGEB.D.AUSST.UNTERS.W.NACHGES.


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[redacted] MARGARETA                                GESCHLECHT: W GEBR.:  2.11.50

CHARLOTTENBURG KLINIKUM               POLI [redacted]

EINS-DATUM:     I  1.06.82 I 15.06.82 I
MAT-NR:         I  3537026 I  3537704 I
EINS-NR:        I     1.   I     2.   I

EXANTHEM (MAKU I     *     I     *     I
ROETELN         I     *     I           I

VBL             I     *     I     *     I

VBL-HAH-ROE-000I      4/0   I   512/3   I
VBL-KBR-HSI-000I      4/1   I     4/1   I
VBL-KBR-MAS-000I      4/1   I     4/1   I
VBL-KBR-VIZ-000I      4/1   I     4/1   I
VBL-MHH-ROE-000I            I    50/2   I
VBL-RME         I           I POSITIV I

 1.06.82  3537026/01
IN 8 BIS 14 T.EINS.VON                VBL


15.06.82  3537704/02
ERGEB.D.AUSST.UNTERS.W.NACHGES.


--------------------------------------------------------------------------------

[redacted] SAEUGLING                                GESCHLECHT: M GEBR.:          K

CHRISTOPHORUS-KINDERKRHS.             STATION [redacted]

EINS-DATUM:     I 10.06.82 I
MAT-NR:         I  3537494 I
EINS-NR:        I     1.   I

DURCHFALL       I     *     I

STU             I     *     I

STU-FLM         I   RTA000  I
STU-GAZ         I    FEHLT  I
```

Fig. 8. Printout of an interim diagnostic survey: a list of diagnoses. Given in a condensed form using mnemonics, it is designed for internal use only, in order to obtain an actual survey on the course of the diagnostic work. On this basis, diagnostic interpretations and additional remarks can be given for the medical report to the sender

9 Reports

Upon request, data on all the patients given in the list of diagnoses are printed out, including the diagnoses and further remarks, without any abbreviations or mnemonics (Fig. 9). Since especially in virology the diagnosis is characterized by at least two admissions, it is advisable to send a letter containing data on all the previous admissions. Some additional precautions have been introduced into this system: The operator can decide how many of the previous admissions should be printed in the letter.

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      KLINIKUM CHARLOTTENBURG
      POLI MED
      SPANDAUER DAMM 130                              DATUM: 24.06.82

      1000 BERLIN 19


VERBINDLICHEN DANK FUER DIE UEBERWEISUNG DES UNTERSUCHUNGSMATERIALS
IHRER PATIENTIN:
                [redacted]
                GEB:  2.11.50

DATUM - EINSENDUNG:                    I  1.06.82 I 15.06.82 I

EXANTHEM (MAKULOES)                    I      *   I      *   I
ROETELN                                I      *   I          I

VENENBLUT                              I      *   I      *   I

*** HAEMAGGLUTINATIONS HEMMTEST
    VENENBLUT
ROETELN                                I     1:4  I   1:512++I
*** KOMPLEMENTBINDUNGSREAKTION
    VENENBLUT
HERPES SIMPLEX                         I     1:4- I     1:4- I
MASERN                                 I     1:4- I     1:4- I
VARIZELLEN ZOSTER                      I     1:4- I     1:4- I
*** IGM HAEMAGGL.HEMMTEST
    VENENBLUT
ROETELN                                I          I    1:50+ I
*** ROETELN IGM ELISA
    VENENBLUT                          I          I POSITIV I

VIROLOGISCHE DIAGNOSE:
ANSTIEG DER ANTIKOERPER GEGEN ROETELN.
DER NACHWEIS VON ROETELN - ANTIKOERPERN IM ISOLIERTEN IGM
SPRICHT FUER EINE FRISCHE INFEKTION.


MIT KOLLEGIALEN GRUESSEN               -  : TITER IM NORMBEREICH
                                       +  : ERHOEHETER TITER
PROF. DR. MED. K.-O. HABERMEHL         ++ : SIGNIFIKANTER TITERANSTIEG
```

Fig. 9. Printout of a medical report to the sender in clear text without any abbreviations showing current and previous diagnostic results with interpretation and further remarks

Furthermore, the precaution has been taken to print in the letter only the diagnoses of the last report. Remarks such as "PLEASE SEND ANOTHER SAMPLE OF BLOOD" are printed only once. If a second letter concerning the same admission is printed out, this remark does not appear in the letter, but it remains in the file. Finally, the printouts of the medical reports are sorted according to the sender, in order to facilitate delivery of the letters.

Since all information is stored in the integrated system, it is possible to generate a letter at any time. Interim reports can be given or a letter can be generated immediately after the arrival of a sample and before any results are obtained, giving only the message to "get more information" or to "get additional samples". These letters can be printed out immediately after the input of the patients' data. It is very helpful for establishing a successful diagnostic service to maintain good contact with the clinician.

10 Classification of Information

Access to the computer is gained by logging in. Each authorized user has a special log-in code which works in two steps. Different grades of the log-in code determine which parts of the system are accessible to the user. This concerns entering data concerning patients, printing out results, and correcting the data files.

A problem can arise if the visual display screen is left on after logging in. In this case, all information made available to the initial user could be obtained by other persons. This problem can be solved by two procedures. First, after finishing his or her work the user can stop the output of information onto the screen by typing in a code number. The second procedure is based on an automatic switch-off system. If the keybord of a terminal is not used for a given time (e.g., 2 min) access to the terminal is switched off automatically. In order to continue working on this terminal one has to log in again. This system of maintaining classified information has been criticized on the grounds that a consultant is able to examine another consultant's results. In agreement with *Blair* and *Smith* (1981) it should be pointed out, however, that this facility is generally considered beneficial rather than harmful.

11 Statistical Evaluations

The linkage of clinical data, test results, and diagnostic methods allows detailed statistical evaluations to be carried out. Using the dialogue system, various statistical programs can be performed concerning epidemiological surveys, establishment of serum banks, or questions of administration.

At present, the epidemiological survey of viral diseases in the Federal Republic of Germany is performed using this system, based on the results of 49 virus diagnostic laboratories. In cooperation with the Deutsche Vereinigung zur Bekämpfung der Viruskrankheiten (DVV), the results from the various laboratories are submitted on optical merk-sense cards, on tapes or on discs. An epidemiological survey is made every 4 weeks and is the basis for the report of the German laboratories to the WHO.

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Enzyme Immunoassays and Related Techniques: Development and Limitations

STRATIS AVRAMEAS*

1 Introduction

Enzyme immunoassay procedures, first reported in 1971, were initially used to quantitate antigen and subsequently antibody (*Avrameas* and *Gilbert* 1971a, b; *Engvall* and *Perlmann* 1971, 1972; *Van Weemen* and *Schuurs* 1971). The principles on which they were based were similar or even identical to those of radioimmunoassays. They therefore necessitated the use of a solid phase to separate the free antigen or antibody from the specific antigen-antibody complexes and were consequently termed heterogeneous enzyme immunoassays. In 1972, Rubinstein and collaborators (*Rubinstein* et al. 1972) reported an enzyme immunoassay which did not necessitate the use of a solid phase, since it was based on antibody-mediated changes in enzyme activity. This procedure was defined as a homogeneous enzyme immunoassay. As the present stage of development of these techniques only permits their application to small haptens, we shall confine the present review to heterogeneous enzyme immunoassays. However, since in the past few years several reviews and books have described these assays in detail (*Avrameas* 1976; *Engvall* and *Pesce* 1978; *Feldmann* et al. 1976; *O'Beirne* and *Cooper* 1979; *Oellerich* 1980; *Maggio* 1980), we shall only indicate their essential points very briefly here, concentrating mainly on their present development, potentialities, and limits.

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2 Assay Designs

2.1 Quantitation of Antigens

An antigen can be quantitated by competitive or non-competitive procedures. The most commonly used competitive procedure involves competition between the antigen to be determined and a fixed quantity of the same enzyme-labeled antigen, for a limited amount of the corresponding specific antibody. This antibody can be used either after immobilization on a solid phase, or in a solution in which it is allowed to react with the antigen and is then insolubilized by means of a second immobilized antibody directed against the first one.

Two noncompetitive procedures, the immunometric and sandwich assays, are used for antigen quantitation. The immunometric procedure involves an excess of enzyme-labeled antibody and immobilized antigens. The labeled antibody is first incubated with the antigen to be measured, and the immobilized antigen is then added. The sandwich procedure requires the use of an excess of labeled antibody and an immobilized antibody with the same or similar specificity. The antigen to be measured is incubated with the immobilized antibody, the excess antigen eliminated by washings, and the enzyme-labeled antibody added. This procedure can only be used with antigens possessing at least two antigenic determinants.

Whether competitive or noncompetitive procedures are used, the enzyme activity most often measured is the one associated with the solid rather than the liquid phase. This is done after adequate washing of the solid phase. The amount of antigen is calculated from the enzyme activity by reference to a standard curve established with known amounts of antigen and obtained under the same experimental conditions.

When applicable, the sandwich procedure is considered to give the most satisfactory results. The major limitation of this method is the requirement that at least two antigenic determinants be present on the antigen molecule to be estimated. Therefore, in the case of low molecular weight substances like drugs and hormones, the immunometric or competitive assay must be used. Until a short time ago, the latter procedure was almost invariably chosen, but recent results obtained with the immunometric assay seem to indicate that this method also has good potential.

2.2 Quantitation of Antibodies

Antibody can be quantitated by two noncompetitive procedures. The first involves the use of excess immobilized antigen and excess labeled antigen. The immobilized antigen is incubated with the antibody to be measured, the excess antibody eliminated by washings, and the enzyme-labeled antigen added. This procedure is based on the observation that, in most cases, only one of the two or more active antibody sites reacts with the immobilized antigen, thus leaving the other sites accessible to the labeled antigen added secondarily. The second procedure involves the use of excess immobilized antigen and excess enzyme-labeled anti-immunoglobulin antibody. As for the first method, the immobilized antigen is incubated with the antibody to be measured, the excess antibody eliminated by washings, and the enzyme-labeled anti-immunoglobulin antibody added. In both procedures the enzyme activity associated with the im-

mobilized antigen is measured. The most satisfactory results as regards sensitivity were obtained when labeled anti-immunoglobulin antibody rather than labeled antigen was used, although a lower background was often reported with the latter reagent.

Although antibody quantitation by heterogeneous enzyme immunoassays has been successfully and extensively applied in various fields, it should always be remembered that antibodies directed against a given antigen possess different affinities for this antigen. This means that different amounts of antibodies with the same apparent specificities but different affinities will bind to the same antigen. Unlike antigen measurement, antibody measurement in the strict sense may not be possible, and the value obtained with these heterogeneous assays may reflect both the quantity and the affinity constant of the antibody (*Butler* et al. 1978).

3 Immobilization of Antigens and Antibodies

In all heterogeneous enzyme immunoassays, an antigen or an antibody attached to a solid phase should be used at one time or another. Solid-phase immobilization of antigen or antibody is obtained either by covalent binding or by adsorption through noncovalent interactions. Particles of agarose, cellulose, polyacrylamide, and polyacrylamide-agarose are activated by various chemical reagents and used for covalent antigen or antibody attachment. Such covalently coated particles have been successfully used in various immunoassays, the particle being separated from the liquid phase by centrifugation after each incubation and washing step. Alternatively, particles containing magnetic iron oxide can be prepared and separated in a magnetic field, thus avoiding centrifugations (*Guesdon* and *Avrameas* 1977).

Various supports have been tested for physical antigen and antibody adsorption, but plastic carriers such as beads, films, plates, and tubes are the most common and have proved the most successful. Adsorption to such surfaces is normally obtained by electrokinetic, electrostatic, but mainly hydrophobic interactions.

Because antigen or antibody immobilization on plastic surfaces is very simple and plastic carriers are easy to handle, this type of immobilization has led to the development of enzyme immunoassays suitable for large-scale work. Of these, the method using microtitration plates has enabled the elaboration of automatic devices making heterogeneous enzyme immunoassays an attractive alternative to the already existing automatic radioimmunoassays. We recently tested the possibility of replacing microtitration plates with Terasaki plates in several antigen-antibody systems and found that the latter plates were just as suitable for working out satisfactory enzyme immunoassays (*Pateraki* et al. 1981). Terasaki plates require 5–10 μl sample reagent and are as easy to handle as microtitration plates. If in the future automatic devices could be developed, these plates might prove a useful and interesting alternative to microtitration plates.

A few years ago, the major disadvantage of the plastic surfaces was that the adsorbing capacity varied among wells of the same plate. In the plates now available, this serious drawback appears to have been overcome, although not completely. In addition to the fact that only a limited amount of antigen or antibody can be immobilized on plastic surfaces, the main drawback now is that antigen or antibody thus immobilized loses part of its binding capacity in the course of the assay. This raises the

nonspecific signal (background) and concomitantly weakens the specific signal (antigen-antibody reaction). Recently, attempts were made to overcome this difficulty by derivatizing plastic surfaces in order to permit covalent protein binding. The results obtained do not yet permit conclusions on whether such procedures can advantageously replace simple physical absorption on a plastic support. Magnetizable particles bearing covalently attached proteins might be a satisfactory alternative to plastic surfaces, but no systematic investigations have yet been undertaken in this direction.

In addition to specific antigen-antibody binding, heterogeneous enzyme immunoassays always involve various degrees of nonspecific binding of the enzyme conjugate to the coated solid phase. The amount of nonspecific binding is considered to be larger with physically coated hydrophobic solid phases (plastic) than with covalently coated hydrophylic solid phases (cellulose, agarose, polyacrylamide), although no systematic experiments have been carried out to test this. Such nonspecific adsorption can be reduced by including in the medium, during the incubation and washing steps, a nonionic detergent such as Tween 20, either alone or supplemented with a protein such as bovine serum albumin or gelatin. However, it should be realized that even under the best-defined conditions, there will always be nonspecific adsorption of the enzyme-conjugate to the coated solid phase.

Whatever solid phase and immobilization procedure is chosen, the quantity of antigen or antibody fixed to the solid phase is of critical importance to the establishment of an efficient enzyme immunoassay. Coating (whether physical or covalent) at high antigen or antibody concentrations reduces specific binding and enhances nonspecific binding. When the concentration of the antigen or antibody used for coating is too low, the specific binding capacity of the coated solid phase will be small, and consequently the sensitivity of the assay will be low. Thus, it is important to find the optimal coating concentration for each antigen-antibody system under investigation, and when possible, to use the purest antigen and antibody preparations available.

4 Conjugates

The effectiveness of the enzyme antibody or antigen conjugate plays a very important role in the development of enzyme immunoassays. Conjugate effectiveness depends on both the enzyme and the antibody used and on the procedure chosen to link the two.

The enzymes mostly used with success in the various enzyme immunoassays include calf intestinal and *Escherichia coli* alkaline phosphatase, *E. coli* β-galactosidase, horseradish peroxidase, and *Aspergillus niger* glucose oxidase. These enzymes possess a high substrate turnover number, do not lose much of their activity after linkage to the antibody, and remain stable at room temperature throughout the periods usually required for immunoassays. Chromogenic and fluorogenic substrates are available for all four of these enzymes. As regards alkaline phosphatase and β-galactosidase, these can be easily and accurately determined with *p*-nitrophenyl phosphate and *o*-nitrophenyl-β-*D*-galactosidase respectively, using a spectrophotometer, and minute amounts of enzyme can be measured with 4-methylumbelliferyl phosphate or 4-methylumbelliferyl-β-*D*-galactosidase. In the case of peroxidase and glucose oxidase, several chromogenic donors have been used for their spectrophotometric measurement, but the results indicate that *o*-phenylenediamine is the substance that gives the most sensitive

and reliable measurements. It is also possible to estimate these two enzymes fluorometrically, but in this case a decline rather than a rise in fluorescence is observed.

The sensitivity of all immunoassays depends greatly on the avidity of the antibody used. This is even more important in enzyme immunoassays in which conjugation of the antibody with the enzyme sometimes lowers antibody affinity for the antigen, probably due to steric hindrances. The enzyme can be conjugated either with the IgG fraction of the immune serum obtained by DEAE ion-exchange chromatography, or with pure antibodies isolated by immunoadsorption. To reduce nonspecific adsorption, it is preferable, when possible, to use isolated antibodies. In the future, the use of avid monoclonal antibodies obtained from established hybridoma cell lines might obviate the need for polyclonal antibodies isolated by immunoadsorption.

Enzymes can be linked to antibodies by either chemical bonding or noncovalent interaction. To obtain covalent enzyme-antibody linkage, several cross-linking agents have been used, the most common being gluteraldehyde, *m*-periodate, and maleimide derivatives (*Avrameas* et al. 1978). Glutaraldehyde, the most frequently used, reacts with free ϵ-amino groups of proteins and has been used in the preparation of almost all enzyme-antibody conjugates intended for enzyme immunoassays. Periodate, which generates active aldehyde groups in the polysaccharide chains of glycoprotein enzymes, is mainly employed to prepare peroxidase antibody conjugates. *N'*-*N'*-*o*-phenylenedimaleimide and *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, which react with sulphydryl groups, are mostly used to couple antibodies to β-galactosidase, which possesses a large number of free sulphydryl groups.

Except for the two-step glutaraldehyde coupling procedure used to prepare peroxidase antibody conjugates, all the other coupling procedures produce more or less heterogeneous populations of conjugates, some of which are composed of high molecular weight derivatives. However, in heterogeneous enzyme immunoassays, which involve no particular accessibility problem, high molecular weight conjugates (probably containing a large number of enzyme molecules) can be used to advantage, provided they do not raise nonspecific adsorption. Sandwich enzyme immunoassays using covalently prepared conjugates of peroxidase, glucose oxidase, alkaline phosphatase, or β-galactosidase and the same corresponding antibody and chromogenic substrates have shown that these four conjugates possess virtually identical sensitivities and are capable of measuring similarly small quantities of antigen or antibody. However, the best accuracy and reproducibility were obtained with alkaline phosphatase and β-galactosidase, whereas with peroxidase, the procedure was the least time-consuming.

Biospecific interactions have been used for noncovalent enzyme-antibody linkage. The first to be chosen for this purpose was the antigen-antibody interaction, on the basis of the observation that enzyme–anti-enzyme complexes always possess sufficient residual catalytic activity for enzyme detection (*Avrameas* 1969; *Mason* et al. 1969; *Sternberger* et al. 1970). Later reports described enzyme immunoassays using the avidin-biotin and the lectin-sugar systems (*Guesdon* et al. 1979; *Guesdon* and *Avrameas* 1980).

In the avidin-biotin system, biotin is covalently coupled through an active ester to the amino groups of an antibody, and a covalently or noncovalently prepared avidin-enzyme complex is then used to reveal the biotin-labeled antibody. In this system, no polymeric forms of antibody, which may have a diminished antigen binding capacity, are produced and the enzyme only undergoes very slight changes.

In the lectin-sugar system, lectin is first covalently linked to the antibody, and enzyme bearing polysaccharide chains capable of specifically interacting with the marker lectin then serve to reveal the lectin-labeled antibody. This system generally makes use of a native unmodified enzyme, which therefore possesses full enzymatic activity. Several marker substances other than enzymes can also be employed with this system; in particular, the use of erythrocytes as markers has allowed the development of a simple titration procedure which is just as sensitive as enzyme immunoassays.

5 Present Limits of Heterogeneous Enzyme Immunoassays

The sensitivity, specificity, reproducibility, and precision of any heterogeneous enzyme immunoassay depend on its design and even more on the choice of solid phase, antibody, enzyme, enzyme measurement method, and enzyme-antibody conjugate. Some of these points, which constitute the limiting factors of such assays, have been discussed above. The section which follows will therefore be restricted to experiments dealing with sensitive enzyme immunoassays in which the importance of these limiting factors is particularly evident.

The model system selected for the development of sensitive enzyme immunoassays was the measurement of human IgE (*Labrousse* et al. 1982). The sandwich, immunometric, and competitive procedures were therefore compared using IgE or anti-IgE antibody labeled with β-galactosidase and the fluorogenic substrate. It was found that the sandwich assay was about ten times more sensitive than the two others, that its reproducibility was the most acceptable, and that nonspecific adsorption, i.e., the attachment of the β-galactosidase conjugate to the coated surface, was the least pronounced.

Various conjugates were tested in an attempt to raise the specific signal and reduce the nonspecific signal obtained with the sandwich procedure. Conjugates based on the biotin-avidin interaction gave the highest amplification of the specific signal, but a parallel simultaneous increase in the nonspecific signal was also noted. Similar results were obtained with all the other conjugates examined. It should therefore be stressed that, independent of the conjugate or solid phase used, heterogeneous enzyme immunoassays always involve nonspecific adsorption to a degree depending on the area of the coated surface used in the assay. At present, this nonspecific adsorption constitutes one of the two main factors limiting the accurate measurement by enzyme immunoassay of extremely small amounts of antigen.

The second and probably the most important factor limiting the development of sensitive enzyme immunoassays is the nature of the antigen-antibody reaction. In this respect it was found that under well-defined conditions, the same lowest concentration of IgE was measured (approximately 0.05–0.1 ng IgE/ml) whether the substrate was fluorogenic or chromogenic. This finding was independent of the fact that with the fluorogenic substrates as few as 5000 molecules of β-galactosidase in solution could easily be measured under the same experimental conditions. These and other similar experiments indicated that in an immunoassay using a highly sensitive marker substance, the limiting factor is not detection of the label but rather the extent of antibody interaction with the antigen. It is possible that in the future, the use of highly avid monoclonal antibodies will enable this crucial difficulty to be overcome at least in part.

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Detection of Antiviral IgM Antibodies and its Problems - A Review

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1 Introduction

The presence of antibody activity in various serum immunoglobulin fractions was discovered as early as the 1930s (*Heidelberger* and *Pedersen* 1937; *Kabat* 1939), but it took another 20 years before the specific characteristics of antibodies having different molecular weights were recognized (*Stelos* 1958). Subsequent studies conducted in experimental animals and in man using various antigens, including viruses, indicated that the first immunoglobulins which appear after a primary antigenic stimulus are of the IgM class. These then disappear rapidly, usually within a few weeks, and are replaced by IgG antibodies that persist for a longer period. IgG is also the predominant antibody class in secondary immune responses (*Bauer* and *Stavitsky* 1961; *Uhr* and *Finkelstein* 1963; *Svehag* and *Mandel* 1964). The precedence of IgM antibodies observed in these early studies was later criticized as being a methodological error (*Osler* 1978), and in some more recent studies IgM and IgG (and IgA) antibodies have

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been shown to appear almost simultaneously but with individual variation (*Cradock-Watson* et al. 1979a; *Halonen* et al. 1979a).

However, the transient nature of the IgM antibody response seemed to hold true for most primary viral infections, and *Schluederberg* (1965) suggested that the determination of specific IgM antibodies might provide a valuable method for recognizing recent viral infections as well as for differentiating between the primary and secondary immune responses. Thereafter, various methods for the determination of specific IgM antibodies applicable to routine laboratory diagnosis were developed, at first for rubella serology where the need for rapid diagnosis was most obvious (*Banatvala* et al. 1967; *Baublis* and *Brown* 1968; *Cohen* et al. 1968; *Vesikari* and *Vaheri* 1968), and subsequently for many other viruses with diagnostic significance.

As indicated above, the use of IgM antibody determination in diagnostic virology is based on the hypothesis that an acute viral infection can be diagnosed by demonstrating the presence of (and excluded by demonstrating the absence of) specific IgM antibodies in a single serum specimen taken at an early stage of the illness. This would enable a rapid laboratory diagnosis and provide a considerable advantage over classical serology, which is based on the demonstration of a significant rise in titer between paired serum specimens taken 1–3 weeks apart. For this hypothesis to be valid, the IgM antibody response should be:

1. Measurable with adequate reliability and sensitivity
2. Biologically specific
3. Constant
4. Transient
5. Sufficiently uniform

2 Methods Used for Determination of IgM Antibodies

It is evident that the first qualification point in the above list must be fulfilled before the others can even be studied, and until now the vast majority of publications dealing with IgM antibody determination have covered the technical aspects. Since the introduction of the first diagnostic applications of IgM antibody determination, the variety of methods applied has increased steadily (Table 1). Furthermore, several modifications of these techniques as well as combinations of methods based on different principles are used.

2.1 Comparison of Antibody Titers Before and After Inactivation of IgM Antibodies

These methods are based on the ability of mercaptans, e.g., 2-mercaptoethanol, to split the IgM molecule into immunologically inactive parts by breaking the disulphide bonds between the polypeptide chains. The serum specimens both treated and untreated, are tested by standard serological methods, and the presence of IgM antibodies is indicated by a significant decrease in titer in the treated specimen. The method is simple and it was also one of the first suggested for routine purposes (*Banatvala* et al. 1967). However, to obtain a fourfold or greater (the usual significance level in serology) difference between the titers of treated and untreated serum, at least 75%

Table 1. Methods used for IgM antibody determination

1. Comparison of antibody titers before and after inactivation of IgM antibodies
Alkylation-reduction by mercaptans
2. Antibody determination from the isolated IgM fraction after physicochemical separation of IgM
Sucrose density gradient fractionation
Gel filtration
Ion-exchange chromatography
Affinity chromatography
3. Antibody determination from serum after removal of IgG (and IgA) antibodies
Protein A absorption
Anti-IgG (and anti-IgA) absorption
4. Indirect solid-phase immunoassays using labeled anti-human IgM antibodies
Immunofluorescence
Immunoperoxidase assay
RIA
EIA
5. Solid-phase anti-IgM assays
RIA
EIA
Solid-phase immunosorbent hemagglutination inhibition
Solid-phase immunosorbent hemadsorption
6. Other assays used for the determination of IgM antibodies
Immunodiffusion
Counterimmunoelectrophoresis
Radioimmunodiffusion
Radioimmunoprecipitation
Anti-IgM blocking RIA
Latex-IgM agglutination
Microimmunobead-IgM hemagglutination reduction
Anti-IgM hemagglutination

of the total antibodies have to be of the IgM class. This is usually true only during the very early stages of an infection, and in their comparative study on rubella diagnostis, *Forghani* et al. (1973) clearly demonstrated the low sensitivity of this method. Therefore, routine use of this method has been restricted almost invariably to the diagnosis of tick-borne encephalitis, which shows a marked precedence of the IgM antibody response (*Kunz* and *Hofmann* 1971). On the other hand, treatment with 2-mercaptoethanol is commonly used as a control step in connection with other techniques, e.g., sucrose density gradient fractionation (*Caul* et al. 1974) and protein A absorption (*Roggendorf* et al. 1976).

Since 2-mercaptoethanol, the most commonly used mercaptan, has an obnoxious odor that complicates its laboratory use, it is now often replaced by dithiothreitol, which has comparable properties in the inactivation of IgM antibodies (*Okuno* and *Kondelis* 1978).

2.2 Antibody Determination from the Isolated IgM Fraction After Physicochemical Separation of IgM

Because IgM has a higher sedimentation coefficient (19S) than other immunoglobulins (7–11S), IgM antibodies can be separated from other antibodies by rate-zonal centrifugation. In 1968 *Vesikari* and *Vaheri* introduced *sucrose density gradient fractionation* followed by the hemagglutination-inhibition (HI) test of the fractions for routine diagnosis of rubella infections, and during the subsequent years several modifications of the original procedure were published (*Best* et al. 1969; *Desmyter* et al. 1971; *Forghani* et al. 1973; *Caul* et al. 1974). Most authors simplified the original method by collecting and testing only one IgM-containing fraction (*Forghani* et al. 1973; *Caul* et al. 1974) instead of the 12–14 fractions used by *Vesikari* and *Vaheri* (1968). In contrast, *Dibbert* (1976) preferred to test 12 fractions because the titer profile of the fractions shows characteristic patterns at various periods after recovery, and thus gives more information than one IgM peak titer value.

Instead of the HI test, the isolated IgM fractions can be tested with other suitable serological tests including, e.g., the complement-fixation test (*Abe* and *Inouye* 1979), the neutralization test (*Schmidt* and *Lennette* 1975), immunoelectrophoresis (*Cohen* 1978), passive hemagglutination (*Al-Nakib* 1980), and radioimmunoassay (*Locarnini* et al. 1977; *Hawkes* et al. 1980), which have made possible the application of sucrose density gradient fractionation in the diagnosis of a variety of viral infections.

One problem in sucrose density gradient fractionation is how to control the purity of the IgM fractions, because trailing of IgG antibody into the IgM fractions may contaminate these and lead to false-positive results. This can happen especially when the IgG titers are high (*Forghani* et al. 1973), the serum has rheumatoid factor (RF) activity (*Gispen* et al. 1975), or the serum has been heat inactivated prior to fractionation (*Pattison* et al. 1976). Fraction purity has usually been controlled either by pretesting the fractions using immunodiffusion (*Best* et al. 1969; *Scott* et al. 1972; *Forghani* et al. 1973) or by treating a portion of each fraction with 2-mercaptoethanol (*Field* and *Murphy* 1972; *Edelman* and *Pariyanonda* 1973; *Caul* et al. 1974). Both methods have been criticized. The sensitivity of immunodiffusion may be too low to detect small amounts of contaminating antibody, causing false-positive results when sensitive assays are used to test the fractions (*Roggendorf* et al. 1976). In addition, after 2-mercaptoethanol treatment the reduced subunits may reaggregate and lead to a false-negative result (*Forghani* et al. 1973). Furthermore, animal studies have shown that IgG is not totally resistant to 2-mercaptoethanol but can also be degraded, especially when the IgG concentration is low (*Capel* et al. 1980).

Comprehensive studies on the routine use of sucrose density gradient fractionation have, however, shown that the method has good reliability and sufficient sensitivity (*Dibbert* 1976; *Caul* et al. 1978). It has therefore been the most commonly used technique in rubella diagnosis and was the standard for comparison when new IgM antibody tests were developed (*Denoyel* et al. 1981; *Field* et al. 1980; *Meurman* et al. 1977; *Mortimer* et al. 1981a). Sucrose density gradient fractionation is, however, a rather laborious technique, and the limited capacity of ultracentrifuges makes it inconvenient for routine screening of large numbers of specimens. A considerable improvement was the recent introduction of vertical rotors with reorienting gradients, which made it possible to reduce the centrifugation time to 2 h from the 16–20 h

required with swinging-bucket rotors (*Wolf* et al. 1979). This new technique has already been successfully applied to the diagnosis of rubella (*Luton* and *Ridgway* 1979), hepatitis A (*Frösner* et al. 1979), and hepatitis B virus infections (*Swenson* et al. 1981).

An alternative method of separating IgM antibodies from serum is gel filtration, which is based on the larger size of IgM (mol. wt. 900000) compared with other classes of immunoglobulin (mol. wt. 150000–400000). The most commonly used method has been *column chromatography* with Sephadex G-200 (*Gupta* et al. 1971) or Bio-Gel A5M (*Bürgin-Wolff* et al. 1971). The elution patterns of the various gels available and their suitability for IgM diagnosis have been extensively studied by Pattison and co-workers (*Pattison* and *Mace* 1973, 1975b; *Pattison* and *Jackson* 1978; *Morgan-Capner* et al. 1980). Adequate separation of IgM and IgG antibodies was also obtained with Ultrogel AcA 34 and Sephacryl S-300 gels. In addition to column chromatography with columns of, e.g., 33 × 2.6 cm minicolumns (*Pyndiah* et al. 1977) and thin-layer chromatography (*Jansen* 1975) have also been used.

In gel filtration the purity of the fractions presents a similar problem as in sucrose density gradient fractionation, because with neither method can total separation of the immunoglobulin classes be achieved (*Sugg* et al. 1979). As control steps with gel filtration, both immunodiffusion (*Ishii* et al. 1968) and 2-mercaptoethanol treatment (*Morgan-Capner* et al. 1980) have been used.

A further drawback of the gel filtration technique is the resulting tenfold dilution of the specimen, which sometimes necessitates concentration of the collected IgM fraction prior to testing (*Bürgin-Wolff* et al. 1971; *Monath* 1971; *Pead* 1974). However, gel filtration is practically as reliable and sensitive a method as sucrose density gradient fractionation. Good results have been obtained, e.g., in diagnosis of postnatal rubella virus infections (*Pattison* and *Mace* 1975a; however, in the diagnosis of congenital rubella, gel filtration was shown to be less sensitive than sucrose density gradient fractionation (*Pattison* et al. 1978).

As with sucrose density gradient fractionation, gel filtration also has limited capacity and is therefore not optimal for large-scale applications. Using rigid gels, e.g., Sephacryl S-300, which allow a higher flow rate, more specimens can be fractionated per column per day than with Sephadex G-200 (*Morgan-Capner* et al. 1980). An even more rapid eluant flow rate can be used with chromatography on controlled porous glass because glass is not at all compressible (*Frisch-Niggemeyer* 1975). Another solution to the capacity problem is the use of a multichannel apparatus for the simultaneous handling of several columns (*Inouye* and *Kono* 1981). With this apparatus, automatic concentration of the collected fractions is also possible.

Other, less frequently applied methods of separating IgM from serum include *ion-exchange chromatography* (*Nagy* and *Mezey* 1977; *Johnson* and *Libby* 1980), which is based on the differential binding of the immunoglobulin classes to anion-exchange resins (e.g., QAE-Sephadex A-50), and *affinity chromatography* on columns of anti-human IgM covalently bound to sepharose beads (*Barros* and *Lebon* 1975).

2.3 Antibody Determination from Serum After Removal of IgG (and IgA) Antibodies

In 1966 *Forsgren* and *Sjöquist* discovered that *protein A*, a cell wall component of some *Staphylococcus aureus* strains, binds to the Fc part of the human IgG molecule.

Ankerst et al. (1974) applied this finding to viral diagnosis by suggesting that detection of specific antibodies in sera absorbed with protein A-containing staphylococci could be used as an indication for a recent rubella virus infection. However, absorption does not remove all IgG antibodies from high-titer sera, partly because subclass IgG_3, which constitutes about 5% of total IgG, is not absorbed (*Kronvall* and *Williams* 1969). Furthermore, a major portion of the IgA antibodies remains after absorption, and these can persist for long periods after infection (*Hornsleth* et al. 1975; *Halonen* et al. 1979b; *Schmitz* and *Haas* 1972). For this reason 2-mercaptoethanol controls proved necessary in positive specimens (*Roggendorf* et al. 1976; *Handsher* and *Fogel* 1977). Somewhat better results were obtained when purified protein A coupled to sepharose, instead of whole staphylococci, was used for absorption. But with both modifications the sensitivity of the test was inferior to that of sucrose density gradient fractionation (*Roggendorf* et al. 1976; *Field* et al. 1980). A factor which lowers the sensitivity is that the absorption is not specific but also removes up to 60% of the IgM antibodies (*Roggendorf* et al. 1976; *Field* et al. 1980).

An alternative method for removing IgG antibodies from serum was introduced by *Schmitz* et al. (1975), who *precipitated IgG* by means of anti-human IgG. Using this method, the IgG was almost totally removed, but about 40% of the IgM was also lost, whereas a major portion of the IgA antibodies remained.

A combination of these methods was introduced by *Geisen* et al. (1979), who used protein A absorption followed by an absorption with anti-human IgG and anti-human IgA, both coupled to controlled porous glass. This improvement increases the efficacy and specificity of the absorption but due to the relatively low sensitivity of the absorption method it is only recommended for screening tests (*Field* et al. 1980). In addition to its use in rubella diagnosis combined with the HI test, the absorption method has also been combined with radioimmunoassay in hepatitis A diagnosis (*Bradley* et al. 1979), and with the neutralization test in enterovirus diagnosis (*Reiner* and *Wecker* 1981).

2.4 Indirect Solid-Phase Immunoassays Using Labeled Anti-Human IgM

In all these tests the general principle is that test serum is incubated with viral antigens bound onto a solid-phase support, and specific IgM antibodies bound to the antigen are subsequently detected with anti-human IgM antibody labeled with a suitable marker (Fig. 1a).

The *indirect immunofluorescence* test, in which organic fluorochromes (mostly fluorescein isothiocyanate) are used as a marker, is the oldest of these assays, and it was also the first to be applied for determination of antiviral IgM antibodies (*Baublis* and *Brown* 1968; *Cohen* et al. 1968; *Hanshaw* et al. 1968). Infected cells fixed on microscope slides are usually used as antigen, but with noncultivable viruses, sections of infected organs can equally well be utilized (*Brzosko* et al. 1975). The use of immunofluorescence is restricted to laboratories with cell-culture facilities and requires special expertise, since only certain virus-cell culture systems are suitable for the production of antigen for immunofluorescence, and optimal handling of the cultures is necessary (*Haire* et al. 1972).

In sera with a high IgG titer, the IgM reactivity can be blocked by competitive inhibition of IgM antibodies (*Cohen* et al. 1967; *Schmitz* and *Haas* 1972; *Cradock-*

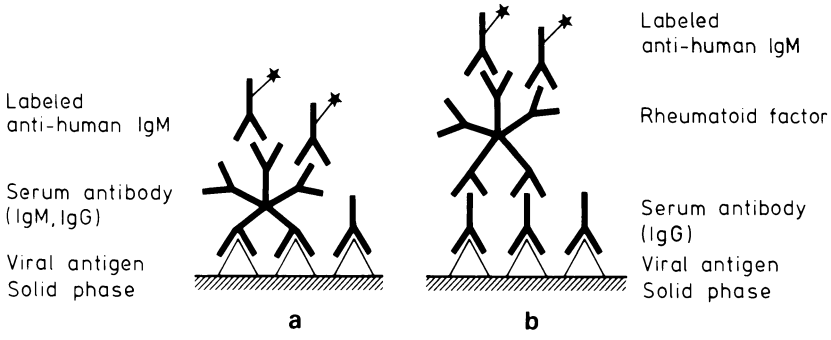

Fig. 1. Indirect solid-phase immunoassay. a) The test principle of indirect solid-phase immunoassays b) The mechanism of RF interference in these assays

Watson et al. 1972, 1976). Therefore, many authors have obtained satisfactory sensitivity only when the immunofluorescence test is performed using separated IgM fractions (*Cradock-Watson* et al. 1972, 1976; *Dall* et al. 1980) or in sera in which the IgG concentration has been reduced by precipitation anti-IgG (*Gispen* et al. 1975; *Hekker* et al. 1979), by ion-exchange chromatography (*Hornsleth* et al. 1974, 1975), or by protein A absorption (*Skaug* and *Tjøtta* 1974; *Skaug* and *Gaarder* 1978). Competition between IgG and IgM antibodies was not, however, observed by some authors (*Kurtz* 1974; *Sheinbergas* et al. 1978).

The reading of immunofluorescence slides requires great skill and experience. On the other hand, an experienced microscopist can eliminate false-positive results by differentiating the morphological patterns of specific and nonspecific fluorescence, a possibility that does not exist in radioimmunoassays and enzyme immunoassays.

Due to the many factors complicating the immunofluorescence test, variable results have been reported. However, in its advanced forms and in experienced hands the immunofluorescence test is a sensitive and reliable method. For example, in a comparative study on rubella diagnosis, *Pattison* et al. (1978) showed that immunofluorescence performed on fractionated sera was more sensitive than sucrose density gradient fractionation followed by the HI test.

The *immunoperoxidase assay* also uses infected cells as antigen, but with horseradish peroxidase-conjugated anti-human IgM as an indicator. With the addition of hydrogen peroxidase and a color-changing indicator, a characteristic granular staining can be observed in cells where the conjugate has been bound. The immunoperoxidase assay, which has only infrequently been applied to the detection of IgM antibodies (*Gerna* and *Chambers* 1977), is generally comparable with the immunofluorescence test, but the slides can be examined under an ordinary light microscope.

In the first *radioimmunoassays* (RIAs) for viral antibodies, infected cell monolayers (*Hayashi* et al. 1972) or infected cells fixed on cover slips (*Hutchinson* and *Ziegler* 1972) were used. However, by exploiting the observation of *Catt* and *Tregear* (1967) that proteins can be nonspecifically adsorbed onto plastics, the use of antigen suspensions in the form of purified virions (*Kalimo* et al. 1978), viral subunits (*Arstila* et al. 1977; *Halonen* et al. 1979a), crude cell lysates (*Knez* et al. 1976), infected allantoic fluid (*Charlton* and *Blandford* 1975), commercial complement-fixing or hemagglutinating antigens (*Kangro* et al. 1978; *Kangro* 1980), or extracts of organs

from infected animals (*Wolff* et al. 1981) became possible. The antigens are usually bound by passive adsorption to a suitable solid-phase support, e.g., polyvinylchloride (*Knez* et al. 1976) or polystyrene (*Dörries* and *ter Meulen* 1980) microtiter plates, polystyrene beads (*Meurman* et al. 1977), or polystyrene tubes (*Daugharty* et al. 1973). However, fixation with acetone, ethanol, or formalin is sometimes included (*Knez* et al. 1976; *Schmitz* et al. 1977a; *Kangro* et al. 1978). An alternative method is to bind the antigens indirectly to the solid phase by first coating the solid phase with a specific antibody which then immunologically binds the antigen (*Daugharty* et al. 1973; *Yolken* et al. 1978).

Using control antigen from uninfected cells or from other sources prepared by procedures similar to those used for the corresponding test antigens, false-positive reactions caused by possible anticellular or antinuclear antibodies can be mostly eliminated. With purified antigens, however, the preparation of comparable control antigens is often impossible, and the results must be evaluated by comparison with a negative control serum (*Meurman* et al. 1977) or with results obtained after blocking by hyperimmune animal serum (*Frisch-Niggemeyer* 1982).

In most RIAs the specific antibodies are detected utilizing ^{125}I-labeled anti-human immunoglobulin antibodies. [^{125}I] can be incorporated effectively into immunoglobulin molecules to high specific activity without markedly affecting their immunological activity and specificity (*Hunter* and *Greenwood* 1962; *Bolton* and *Hunter* 1973).

The advantages of RIAs are the high sensitivity, the possibility of using crude or commercial antigens, and the potential for automation of the test. Competitive inhibition of IgM reactivity by IgG antibodies, a common drawback in immunofluorescence, has been demonstrated in RIA only in experiments using isolated serum fractions (*Knez* et al. 1976), but not when whole serum has been tested (*Knez* et al. 1976; *Meurman* et al. 1977; *Kangro* et al. 1978; *Arvin* and *Koropchak* 1980). In addition, the sensitivity of RIA has been shown to be higher than that of sucrose density gradient fractionation (*Meurman* et al. 1977), gel filtration (*Kangro* et al. 1981), and immunofluorescence (*Cradock-Watson* et al. 1979b). The disadvantages of RIA, on the other hand, are the expensive equipment required, possible radioactive hazards, and the short shelf-life of the iodinated immunoglobulins, which can only be used for 2–4 months.

The *enzyme immunoassays* (EIAs) first applied to the detection of antiviral IgM antibodies by *Voller* and *Bidwell* (1976) are principally similar to RIAs, but in this case the anti-human immunoglobulin antibodies are labeled with enzyme molecules, most commonly with alkaline phosphatase or horseradish peroxidase. Polystyrene microtiter plates (*Voller* and *Bidwell* 1976) are usually used as the solid phase, but polyvinylchloride plates (*Yolken* et al. 1978; *McLean* et al. 1980), polystyrene beads (*Hofmann* et al. 1979), and polystyrene cuvettes (*Leinikki* et al. 1978) have also been used. The antigen preparations used in EIA are identical to those used in RIA. The sensitivity and specificity of these two assays are comparable, although competition between specific IgM and IgG antibodies, not observed in RIA, has been reported with EIA (*Heinz* et al. 1981). The EIA procedure is somewhat more complicated than that for RIA because an additional reaction with the substrate is required before the bound anti-human IgM can be measured. However, EIA offers obvious advantages over RIA. The most important ones are the stability of the labeled anti-

human immunoglobulin antibodies which are commercially available and the fact that expensive equipment is not required. Thus EIAs can be performed in practically every laboratory where viral diagnosis is carried out. For large-scale testing, the handling of microtiter plates can be automated and the recording of results can be accomplished by means of multichannel spectrophotometers which read and print out the results directly from the microtiter plates.

In all these indirect immunoassays, RF, an anti-IgG antibody of the IgM class, may cause false-positive results if sufficient amounts of both specific IgG antibodies and RF are present (*Shirodaria* et al. 1973; *Meurman* and *Ziola* 1978; *Salonen* et al. 1980; *Vejtorp* 1980). This interference will be discussed in detail later in this article.

2.5 Solid-Phase Anti-IgM Assays

In this new type of assay IgM antibodies are separated from serum by immunoadsorption to anti-human IgM bound to a solid-phase support, e.g., a microtiter plate. The adsorbed IgM is then allowed to react with the specific viral antigen. Those antigens bound by specific IgM antibodies are subsequently detected by other iodinated or enzyme-labeled antibodies (Fig. 2a) or measured directly if the antigens are labeled either radioactively or with an enzyme (Fig. 2b). Alternatively, when the antigen is a hemagglutinating virus, red blood cells can be used as an indicator to produce HI or hemadsorption.

This test principle was first used by *Price* (1977) in an RIA for IgM antibodies to *Mycoplasma pneumoniae.* The first application of the solid-phase anti-IgM test in viral diagnosis was the hepatitis A virus IgM antibody RIA by *Flehmig* (1978). An EIA was then reported by *Duermeyer* and *van der Veen* (1978), followed by several modifications for the detection of IgM antibodies to hepatitis A (*Møller* and *Mathiesen* 1979; *Roggendorf* et al. 1980; *Decker* et al. 1981; *Meurman* et al. 1981) and hepatitis B core antigens (HB_cAg) (*Gerlich* and *Lüer* 1979; *Tedder* and *Wilson-Croome* 1980; *Kryger* et al. 1981; *Lemon* et al. 1981; *Mortimer* et al. 1981b). Until now the diagnosis of viral hepatitis has been the main area of application of these tests, and commercial test kits are also available (*Decker* et al. 1981). Subsequently, analogous RIAs and EIAs have been developed for the detection of IgM antibodies to rubella virus (*Mortimer* et al. 1981a; *Vejtorp* 1981; *Diment* and *Chantler* 1981), flaviviruses (*Roggendorf* et al. 1981b; *Heinz* et al. 1981; *Burke* and *Nisalak* 1982), and the herpes group of viruses (*Tedder* et al. 1981; *Yolken* and *Leister* 1981).

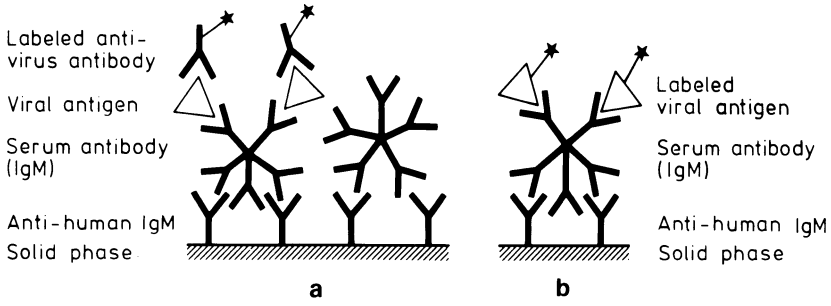

Fig. 2. Solid-phase anti-IgM assay. The test principle of solid-phase anti-IgM assay a) using unlabeled antigen and labeled specific antibody, and b) using labeled antigen

In all of the above-mentioned assays for antiviral IgM antibodies, unlabeled antigens were used, and these were then finally detected by iodinated or enzyme-labeled specific antibodies. A new modification was introduced by *Schmitz* et al. (1980), who developed a cytomegalo virus IgM antibody assay using viral antigen labeled with horseradish peroxidase. By using enzyme-labeled antigens, the test can be shortened by one incubation step, and the possibilities of interference by RF decrease (*Schmitz* et al. 1980). However, labeling antigens is considerably more difficult than labeling immunoglobulins, and therefore only a few assays using enzyme-labeled antigens have been developed so far (*Doerr* et al. 1980; *Van Loon* et al. 1981a, b).

A somewhat complicating factor at present is the confusing nomenclature of these assays. Many authors have given a name of their own to the assay published, and in addition to "solid-phase anti-IgM assay" (*Vejtorp* 1981), designations such as "direct immunoassay" (*Price* 1977; *Van Loon* et al. 1981a; *Decker* et al. 1981), "reverse immunoassay" (*Gerlich* and *Lüer* 1979; *Denoyel* et al. 1981; *Meurman* et al. 1981), "IgM antibody capture assay" (*Mortimer* et al. 1981a; *Burke* and *Nisalak* 1982), "double sandwich IgM assay" (*Naot* et al. 1981), and "enzyme-labeled antigen IgM assay" (*Schmitz* et al. 1980) have been used with RIA and/or EIA applications.

The solid-phase anti-IgM assays have proven to be very specific and sensitive. They have been more sensitive than assays based on sucrose density gradient fractionation (*Mortimer* et al. 1981a; *Roggendorf* et al. 1980), and about equally sensitive to (*Schmitz* et al. 1980; *Vejtorp* et al. 1979; *Yolken* and *Leister* 1981) or more sensitive than (*Heinz* et al. 1981; *Roggendorf* et al. 1981b) corresponding indirect immunoassays. Because the first step in these assays leads to the separation of IgM antibodies from other serum components, competition between IgG and IgM does not occur. Therefore, unlike in indirect immunoassays, the sensitivity of the assay is not influenced by the ratio of antigen-specific IgG to IgM. However, in solid-phase anti-IgM assays specific IgM antibodies compete with other IgM molecules for the binding sites on the solid phase, and therefore the sensitivity of the test is influenced by the ratio of specific IgM antibodies to total IgM (*Heinz* et al. 1981; *Vejtorp* 1981).

A solid-phase anti-IgM assay using red blood cells as an indicator was developed by *Krech* and *Wilhelm* (1979). They performed a rubella virus HI test in microtiter plate wells to which IgM antibodies were bound by solid-phase anti IgM. When specific IgM antibodies were present, viral antigen was bound to these and resulted in HI, whereas in the absence of specific antibodies free antigen caused hemagglutination. An analogous assay has also been developed using polystyrene particles coated with anti-human IgM as the solid phase (*Doerr* 1979; *Doerr* et al. 1980). However, this is hardly advantageous since centrifugation steps are required for washing the particles, and the assay is thus more laborious than the test using microtiter plates.

By changing the reagents, including the diluents, the amount of hemagglutinin, and the concentration of erythrocytes, new modifications of the original test by *Krech* and *Wilhelm* (1979) were developed in which the positive reaction showed up as hemadsorption instead of HI (*Van der Logt* et al. 1981; *Denoyel* et al. 1981; *Goldwater* and *Banatvala* 1981). The sensitivity of these assays was shown to be comparable to that of sucrose density gradient fractionation followed by HI, or to that of indirect immunofluorescence (*Krech* and *Wilhelm* 1979; *Van der Logt* et al. 1981; *Denoyel* et al. 1981). According to one study, the hemadsorption modification was more sensitive than the original HI assay of Krech and Wilhelm (*Van der Logt* et al. 1981).

The advantage of these assays is that with the exception of anti-human IgM antibody no reagents other than those used in conventional HI tests are required. Interference by RF has not been observed (*Krech* and *Wilhelm* 1979; *Van der Logt* et al. 1981; *Denoyel* et al. 1981; *Goldwater* and *Banatvala* 1981). In addition to rubella diagnosis, the solid-phase anti-IgM hemadsorption tests have also been adapted to the detection of IgM antibodies to mumps virus (*Van der Logt* et al. 1982) and to influenza B virus (*Goldwater* et al. 1982).

As a whole, the advantages of the solid-phase anti-IgM assays over the indirect immunoassays are the lack of competition between IgG and IgM, a somewhat higher sensitivity, and above all the decreased interference by RF. This latter point will be discussed in detail later in this article. A disadvantage, on the other hand, is the need for conjugated antiserum or antigen for each antiviral IgM antibody to be tested when the RIA or EIA tests are used. Also, the parallel testing for IgG and IgM antibodies is not possible with solid-phase anti-IgM assays.

2.6 Other Assays Used for the Determination of IgM Antibodies

This group of assays contains some old and, for IgM determination, less frequently used methods, and some recent tests making use of new applications of anti-human IgM antibody.

Immunodiffusion (*Schmidt* et al. 1968, 1973) and counter-immunoelectrophoresis (*Minor* et al. 1979) have been used for the detection of IgM antibodies to coxsackieviruses. In these tests, serum and viral antigen are allowed to diffuse in agar to form precipitin lines. By using appropriate controls the precipitin lines formed by IgM and IgG antibody antigen complexes can be identified and the presence or absence of specific IgM antibody thereby demonstrated.

Radioimmunodiffusion has been applied by *Ogra* et al. (1968, 1971) for measuring IgM antibodies to polio and rubella viruses. In this test, serum antibodies and anti-human IgM were allowed to form precipitin lines, and the presence of specific antibodies in these precipitates was subsequently demonstrated by autoradiography following incubation with ^{32}P-labeled viral antigen.

Radioimmunoprecipitation has been used to detect IgM antibodies to hepatitis B surface antigen (HB_sAg) (*Lander* et al. 1972). In this test, serum was mixed with ^{125}I-labeled HB_sAg and the resulting IgM antibody-antigen complexes were precipitated by the addition of anti-human IgM. The presence or absence of specific IgM antibodies could be demonstrated by comparing the precipitated radioactivity with the total radioactivity.

An anti-IgM-blocking radioimmunoassay for IgM antibodies to hepatitis A virus has been developed by *Bradley* et al. (1977). Serum immunoglobulins were bound to a solid-phase support by passive adsorption, and the presence of specific hepatitis A virus antibodies was then demonstrated by subsequent incubations with hepatitis A virus antigen and labeled antibody to it. By including an incubation with anti-human IgM antibody before the antigen incubation, the reactivity of IgM anti-viral antibodies could be blocked and their presence demonstrated by the resulting decrease in the binding of the labeled antibody.

A latex-IgM agglutination assay for cytomegalovirus IgM antibody has been developed by *Schmidt* and *Klein* (1980). In this assay viral antigen and serum were

first incubated together. The resulting immune complexes were washed and then incubated with latex beads coated with anti-human IgM antibodies. If immune complexes containing IgM antibody were present, an agglutination of the latex beads was seen.

Microimmunobead-IgM hemagglutination reduction assay for rubella virus IgM antibodies has been described by *Braun* et al. (1981). IgM antibodies were isolated from serum by immunoadsorption to polyacrylamide beads coupled with anti-human IgM. The beads coated with IgM antibodies were then incubated with rubella virus hemagglutinin, and after removal of the beads the reduction in titer of the hemagglutinin was measured. The test was more sensitive than sucrose density gradient fractionation followed by HI and there was no interference by nonspecific factors including RF (*Braun* et al. 1981).

An anti-IgM hemagglutination assay for rubella IgM antibodies has recently been developed by *Traavik* (1982). In this assay erythrocytes were first sensitized with a subhemagglutinating dose of rubella virus hemagglutinin. The sensitized erythrocytes were then incubated with serum antibodies. Finally, the erythrocytes were incubated with anti-human IgM antibody which, if specific IgM antibodies were bound to the antigen on the erythrocytes, created bridges between these IgM molecules resulting in hemagglutination. This anti-IgM hemagglutination assay was more sensitive than sucrose density gradient fractionation and free from interference by RF and other nonspecific factors (*Traavik* 1982).

3 Problems of IgM Antibody Assays

The problems involved in the determination of IgM antibodies and in the interpretation of the test results can be divided into two groups: "technical problems", which are method-dependent; and "biological problems", which can be encountered in whichever method is used.

3.1 Technical Problems

As noted above, every test has its technical problems, but assays based on the same test principle usually also have some common problems. Because indirect immunoassays and solid-phase anti-IgM assays are of the greatest current interest, some problems common to these assays, i.e., the background caused by nonspecific binding and the role of RF, will be discussed.

The sensitivity of an immunoassay is usually highly dependent on the level of background, i.e., the degree of radioactivity, absorbance, etc., observed with negative specimens. A high background also increases the amount of "borderline cases" making positive/negative interpretation more difficult.

3.1.1 Nonspecific Binding

3.1.1.1 Indirect Immunoassays

In indirect immunoassays nonspecific binding correlates with the serum dilution and can therefore be attributed to the nonspecific binding of serum immunoglobulins to

the antigen-coated solid phase. The background is usually higher in IgM antibody assays than in IgG antibody assays, since IgM is a more "sticky" molecule than IgG. The first factor affecting the degree of nonspecific binding is the quality of the antigen used. In general IgM antibody assays require a more purified antigen than IgG antibody assays, but for practical and economic purposes crude antigens such as cell lysates are preferred and used whenever possible. They have been used successfully in assays for IgM antibodies to measles virus (*Forghani* and *Schmidt* 1979), mumps virus (*Meurman* et al., to be published), and herpesviruses (*Knez* et al. 1976; *Arvin* and *Koropchak* 1980; *Kalimo* et al. 1978). However, the use of crude antigens is not possible in all assays, and it has been our experience as well as that of others (*Gravell* et al. 1977; *Forghani* and *Schmidt* 1979) that, e.g., in rubella virus antibody assays, purified antigens are necessary, although *Kangro* et al. (1978) have succeeded in using commercial hemagglutinating antigen. A specific problem is serum with antinuclear or anticellular antibodies, which can show high background in assays using crude cell-derived antigens. Fortunately, these can be detected by the use of appropriate control antigens.

Nonspecific binding of immunoglobulins to the antigen-coated solid phase can be decreased by preincubation of the solid phase with inactive proteins such as bovine serum albumin (*Locarnini* et al. 1977), gelatin (*Kangro* et al. 1978), or normal animal serum (*Meurman* et al. 1977; *Popow-Kraupp* 1981), in order to block the remaining free nonspecific protein binding sites. In our opinion, however, a better way to reduce nonspecific binding is to dilute both serum specimens and conjugates with buffers containing Tween 20 and normal animal serum. The normal serum should preferably be derived from the animal species which has been used for production of the anti-human IgM antibody, but fetal calf serum is usually also suitable. Background reduction can also be achieved by fractionation of the sera (*Dittmar* et al. 1979) or by absorption with protein A (*Hacham* et al. 1980) prior to testing.

3.1.1.2 Solid-Phase Assays

In solid phase anti-IgM assays nonspecific binding at low serum dilutions has also been observed (*Yolken* and *Leister* 1981). Often, however, the assay sensitivity allows such high serum dilutions (up to 1:10000, *Roggendorf* et al. 1980; *Mortimer* et al. 1981b) to be used that nonspecific binding of serum immunoglobulins is no longer a problem. In contrast, a critical step in the assay is the incubation with the antigen. For many antigens, prolonged incubation at a low temperature is optimal (*Mortimer* et al. 1981a; *Meurman* et al. 1981; *Burke* and *Nisalak* 1982), since short incubation results in insufficient specific binding and high temperature results in increased nonspecific binding. However, with some antigens short incubations at 37 °C have been successfully used (*Mortimer* et al. 1981b; *Van Loon* et al. 1981a; *Yolken* and *Leister* 1981). In addition, incorporation of serum in the antigen diluent is effective in decreasing the nonspecific binding. Human serum which is negative for the antibodies tested has been used in concentrations from 1% (*Gerlich* and *Lüer* 1979) to 20% (*Burke* and *Nisalak* 1982), but animal sera may also be suitable (*Schmitz* et al. 1980; *Heinz* et al. 1981; *Yolken* and *Leister* 1981; *Meurman* et al. 1981).

3.1.2 Rheumatoid Factor

3.1.2.1 Indirect Immunoassays

Rheumatoid factor, an anti-IgG antibody of the IgM class can cause false-positive IgM antibody results in immunoassays. In indirect immunoassays the mechanism of interference is that RF attaches to specific IgG antibodies bound to the antigen on the solid phase, and the labeled anti-human IgM antibodies subsequently bind to the RF (Fig. 1b). As a result of this mechanism the interference depends both on the level of RF and on the amount of specific IgG antibodies. If the IgG titer of a serum is low, a high level of RF is needed to cause a false-positive IgM antibody result, whereas with a high specific IgG antibody titer only a relatively low amount of RF is required for interference (*Meurman* and *Ziola* 1978). Because both IgG antibodies and RF are required, dilution of the serum causes a rapid decrease in RF interference. Therefore, dose-response curves of false-positive results caused by RF have a steeper slope than those of the true specific IgM results, and the false-positive IgM titers caused by RF are usually low (Fig. 3). It is obvious that RF interference cannot be controlled by the use of a control antigen (Fig. 3).

Although RF activation has been reported in connection with the acute phase of several viral infections, especially cytomegalovirus infections (*Wagner* et al. 1968; *Knez* et al. 1976), a more serious source of error for IgM antibody determinations

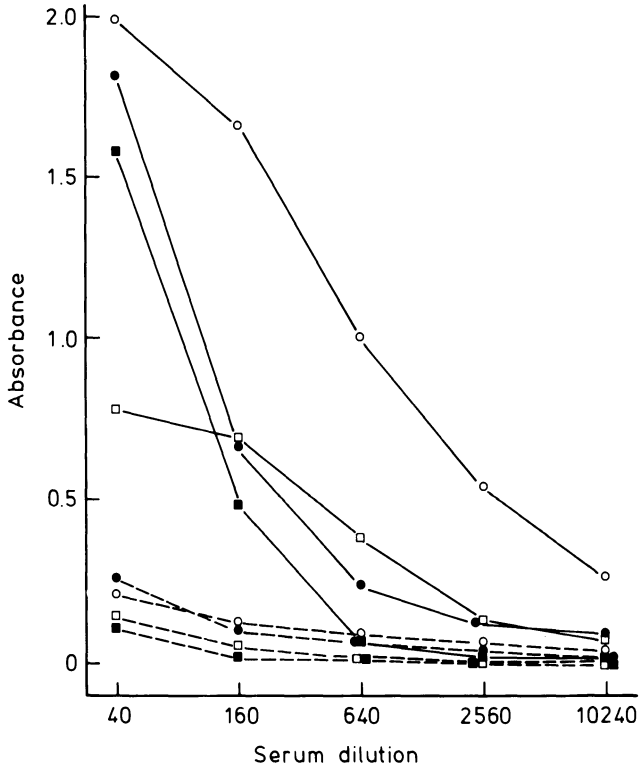

Fig. 3. Representative results of two mumps IgM antibody-positive (○, □) and of two RF-positive mumps IgM antibody-negative (●, ■) sera tested for mumps IgM antibodies by indirect EIA. The sera were tested against both mumps antigen (*solid line*) and control antigen (*dotted line*)

arises in serum from patients with rheumatoid arthritis and related disorders in which continuously high levels of circulating RF are often present. A specific problem, especially in rubella virus diagnosis, arises in tests of serum from pregnant women, because pregnancy often activates the production of low levels of RF (*Meurman* et al. 1978). Likewise, the RF that is occasionally present in the serum of newborns can complicate the diagnosis of congenital infections by means of IgM antibody assays (*Reimer* et al. 1975). The actual rate of false-positive results detected depends greatly on the population studied and on the virus in question. Thus, because IgG antibodies to tick-borne encephalitis are rare, *Hofmann* et al. (1979) estimated the incidence of false-positive tick-borne encephalitis IgM antibody results to be 1 in 1200, whereas in our routine rubella serology during the period 1979–1981, false-positive IgM antibody results were detected in 1 in 125 sera tested.

Since for RF interference IgG antibodies and RF are required, the interference can be eliminated by removal of either RF or the specific IgG antibodies. Removal of IgG antibodies by serum fractionation and subsequent testing of the isolated IgM fractions has proven to be effective in eliminating RF interference (*Robertson* et al. 1977; *Johnson* and *Libby* 1980). Also, methods in which IgG is absorbed by protein B (*Skaug* and *Gaarder* 1978; *Leinikki* et al. 1978) or by anti-IgG (*Gispen* et al. 1975) have been reported to eliminate RF interference. The alternative way to eliminate RF interference is to remove the RF. This can be done by absorption with heat-aggregated IgG (*Meurman* and *Ziola* 1978), glutaraldehyde-polymerized IgG (*Knez* et al. 1976; *Krishna* et al. 1980), or IgG-coated latex particles (*Chantler* et al. 1976; *Vejtorp* 1980). There are somewhat differing opinions concerning the efficacy of these absorption procedures in removing RF interference. Adequate absorption of RF with heat-aggregated IgG was reported by *Meurman* and *Ziola* (1978) and with IgG-coated latex particles by *Vejtorp* (1980), although sera with high RF levels required repeated absorptions. *Leinikki* et al. (1978) found absorption with heat-aggregated or glutaraldehyde-polymerized IgG unsatisfactory and preferred protein A absorption. *Chantler* et al. (1976), on the other hand, found protein A absorption to be insufficient to remove RF interference from sera with high specific IgG antibody titers and proposed a combined absorption with protein A and IgG-coated latex particles. Apparently, an ideal way to avoid false-positive results caused by RF has not been reported, and RF interference is still one of the most important technical problems in the determination of IgM antibodies by indirect immunoassays.

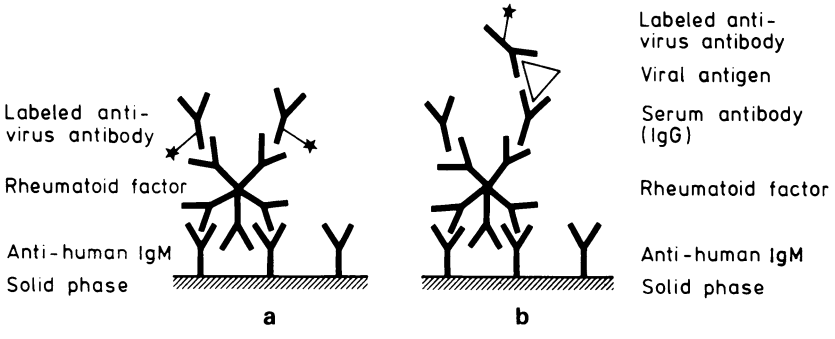

Fig. 4. The mechanisms of RF interference in solid-phase anti-IgM assays. a) Direct binding of labeled anti-virus antibodies and b) interference mediated by specific IgG antibodies and antigen

3.1.2.2 Solid-Phase Assays

In solid-phase anti-IgM assays two possible mechanisms for RF interference exist. In the first mechanism (Fig. 4a), RF bound to anti-IgM on the solid phase will directly bind to labeled antiviral antibodies of the IgG class. In the second mechanism (Fig. 4b), RF binds the specific IgG antibodies, which then subsequently bind to antigen and labeled antibody.

The first mechanism described is the more important of the two. This interference seems to be rare or absent in assays using radiolabeled antibody as an indicator (*Flehmig* et al. 1979; *Tedder* and *Wilson-Croome* 1980; *Meurman* et al. 1981; *Mortimer* et al. 1981a; *Decker* et al. 1981), although some false-positive results have been observed (*Burke* and *Nisalak* 1982). In contrast, most authors using enzyme-labeled indicator antibodies have found false-positive results in sera containing high levels of RF (*Duermeyer* et al. 1979; *Kryger* et al. 1981; *Vejtorp* 1981; *Yolken* and *Leister* 1981). This can be explained by the fact that conjugation with enzymes leads to aggregate formation, and because RF has a higher affinity for aggregated than for

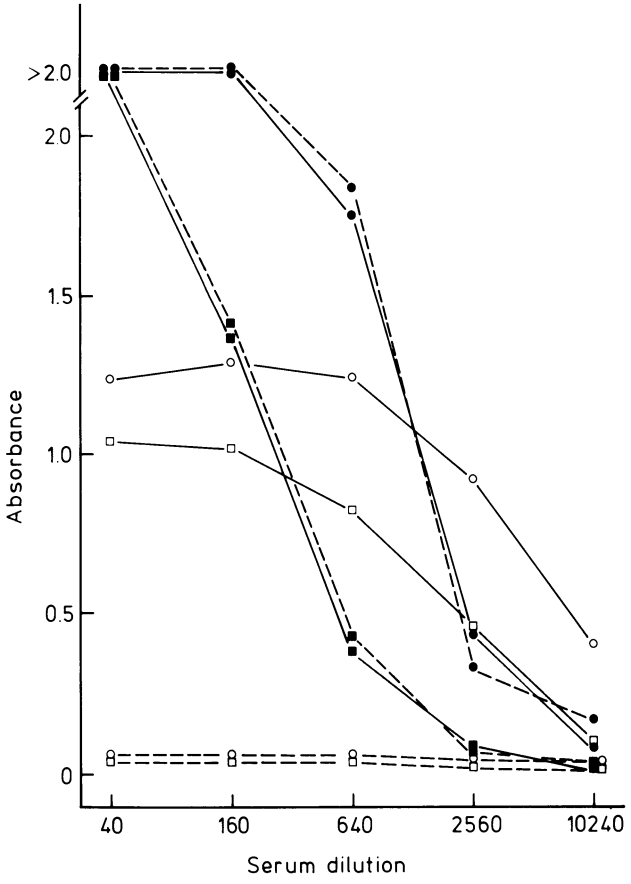

Fig. 5. Representative results of two mumps IgM antibody-positive (○, □) and of two RF-positive, mumps IgM antibody-negative (●, ■) sera tested for mumps IgM antibodies by solid-phase anti-IgM EIA using peroxidase-labeled anti-mumps antibodies as an indicator. Mumps antigen, *solid line*; control antigen, *dotted line*. The same sera were tested as in Fig. 3

native IgG, it will bind the enzyme-labeled immunoglobulins but not the radiolabeled ones. (*Schmitz* et al. 1980). Since this first type of interference is not mediated by specific IgG antibodies and antigen, its presence can be detected and controlled by the use of a control antigen (Fig. 5).

There are several possibilities for overcoming this first type of interference. Apparently, if high serum dilutions are used, interference is likely to occur only in sera having a very high RF titer (*Heinz* et al. 1981). Because RF binds to the Fc part of the IgG molecule, false-positive results can be avoided by using labeled $F(ab')_2$ fragments as the indicator antibody (*Duermeyer* et al. 1979). Of course, in assays using labeled antigen or erythrocytes as an indicator, this type of interference cannot occur. *Gerlich* and *Lüer* (1979) presented a new and simple way of avoiding RF interference by diluting the serum specimens in buffer containing aggregated IgG. The aggregated IgG probably blocks the binding sites of RF and thus prevents false-positive results. The effectiveness of this method was later confirmed by *Vejtorp* (1981). Of course, absorption of the RF can also be used in connection with the solid-phase anti-IgM assays, but this is much more laborious than the use of labeled $F(ab')_2$ fragments or dilution buffers containing aggregated IgG.

The second mechanism of interference (Fig. 4b) can theoretically work in all solid-phase anti-IgM assays, but in practice this type of interference is rare, probably because the affinity of RF for native IgG is low. *Duermeyer* et al. (1979) found only one serum with a high anti-hepatitis A virus IgG titer which gave a false-positive IgM antibody result when tested at a low dilution. *Tedder* and *Wilson-Croome* (1980) detected IgG-mediated RF interference only when aggregated specific IgG antibody was added to RF-positive serum, but not in sera containing RF and specific IgG antibody in native form. This type of interference was also observed when a crude antigen preparation containing performed antigen-antibody IgG in the serum diluent also effectively prevents this IgG-mediated second type of interference.

A third, more complicated mechanism of interference, requiring the combined influence of RF and antinuclear antibodies, has been suggested by *Naot* et al. (1981). The use of labeled $F(ab')_2$ fragments as the indicator antibody and most probably the use of aggregated IgG in the diluent buffer will also prevent interference by this mechanism.

In general, the solid-phase anti-IgM assays are less susceptible to interference by RF than the indirect immunoassays. Furthermore, whereas in indirect immunoassays RF interference can only be suspected, in solid-phase anti-IgM assays it is usually regulated simply by using a control antigen. Because the assay principle in the solid-phase anti-IgM assays does offer some simple possibilities to eliminate the RF interference, there is no doubt that in this respect these assays are clearly superior to the indirect immunoassays which use labeled anti-human IgM antibodies.

3.2 Biological Problems

The demonstration of specific IgM antibodies to a viral antigen can be interpreted as indicating a recent infection caused by the virus in question only if the IgM response is specific – in other words, only if these IgM antibodies are not produced in any other infection. On the other hand, the absence of specific IgM antibodies can be

interpreted as excluding a recent infection only if the IgM response is constant. In addition, variations in the temporal appearance of the IgM antibodies, including the presence of prolonged IgM antibody responses, can cause difficulties in interpreting the significance of the laboratory results in comparison with the actual clinical signs and symptoms.

3.2.1 Cross-Reactions

False-positive IgM antibody results may occur due to cross-reactions between closely related viruses. In studies on experimental togavirus infections in animals, *Westaway* et al. (1974) showed that low levels of IgM antibodies to closely related viruses were detected in primary alpha- and flavivirus infections. In flavivirus infections, after a challenge with a second related virus, IgM memory expression to the primary virus was Detected. Similar cross-reactions have been detected in human alphavirus infections between Ross river and Getah viruses (*Aaskov* and *Davies* 1979) and between the flaviviruses, yellow fever, Japanese encephalitis, St. Louis encephalitis, and different dengue virus serotypes (*Edelman* and *Pariyanonda* 1973; *Vathanophas* et al. 1973; *Wolff* et al. 1981; *Burke* and *Nisalak* 1982). However, the heterologous IgM antibody titers were mostly low compared with the homologous titers, and the IgG antibody response showed much greater type specificity than the IgM antibody response.

In coxsackievirus B infections, heterologous IgM antibody responses to one or more serotypes are common, and sometimes they occur even without the homologous IgM antibody response (*Schmidt* et al. 1968; *Minor* et al. 1979).

In the herpesvirus group, false-positive cytomegalovirus IgM antibody responses have been occasionally detected in patients with Epstein-Barr virus infections (*Hanshaw* et al. 1972; *Schmitz* et al. 1977b; *Hekker* et al. 1979; *Krishna* et al. 1980), although not by all research groups (*Kangro* 1980; *Sundqvist* and *Wahren* 1981; *Van Loon* et al. 1981a). In contrast, heterologous IgM antibody responses between herpes

Table 2. Diagnostic efficacy of complement-fixation and EIA IgG and IgM tests in respiratory infections of young children caused by parainfluenza virus (types 1–3), respiratory syncytial virus, and adenoviruses

Infecting virus	Number of patients	Patient age		Percentage of patients with		
		Median	Range	CF titer increase	EIA IgG titer increase	EIA IgM titer positive
Parainfluenza virus	20	1 year 6 months	5 months–6 years	35	50	25
RSV	27	11 months	2 months–4 years	48	81	63
Adenovirus	21	1 year	2 months–6 years	58	62	62

CF, complement fixation; RSV, respiratory syncytial virus
Paired serum specimens were collected from children with diagnoses confirmed by the detection of viral antigens in nasopharyngeal specimens by radioimmunoassay

simplex and varicella-zoster viruses have not been observed in spite of the occurrence of heterologous IgG antibody responses (*Cradock-Watson* et al. 1979a; *Arvin* and *Koropchak* 1980; *Tedder* et al. 1981).

False-positive mumps virus IgM antibody responses have been reported in connection with parainfluenza virus infections (*Van der Logt* et al. 1982), and heterologous IgM antibody responses between the parainfluenza virus types are also possible. Cross-reactions between mumps and parainfluenza viruses seem to be rare, however, since most authors have not observed such reactions (*Brown* et al. 1970; *Bjørvatn* 1974; *Nicolai-Scholten* et al. 1980).

Heterologous IgM antibody responses are also possible in, e.g., adenovirus infections (*Lehrich* et al. 1966). As with cross-reactivity in general, IgM cross-reactivity can also be a diagnostically useful phenomenon which may enable a group-specific rapid diagnosis in, e.g., influenza virus infections. In instances where cross-reactivity is a drawback, it may be overcome in future using highly purified antigens in the form of intact virions or structural and nonstructural proteins (*Trent* et al. 1976; *Katze* and *Crowell* 1980).

3.2.2 Missing IgM Antibody Responses

Another biological problem is the missing IgM antibody response. According to the general picture, an IgM antibody response is always associated with a primary viral infection, but there are exceptions, and some of the possible situations leading to a missing IgM antibody response include:

1. Infections in young children
2. Local infections
3. Reinfections after natural infection or vaccination
4. Reactivations of latent infections
5. Infections of immunocompromized patients

A group of common viral infections in which IgM antibody determination does not seem to be the optimal diagnostic test are the respiratory infections in young children (Table 2). Young children seem to be poor IgM responders, and *Welliver* et al. (1980) have shown that in respiratory syncytial virus (RSV) infection the IgM response in children aged 1–3 months was markedly weaker than in children aged 3–12 months. A second factor is that respiratory infections, especially parainfluenza virus infections, are of a local nature and may therefore induce too weak a systemic stimulus for an IgM antibody response to be elicited. Similar weak IgM responses have been reported earlier in RSV infections by *Cranage* and *Gardner* (1980), in parainfluenza virus infections by *Smith* et al. (1967) and *Ukkonen* et al. (1980), and in adenovirus infections by *Roggendorf* et al. (1982). A third factor reducing the usefulness of IgM antibody determination in respiratory infections is that these infections may be reinfections, or antibodies to related parainfluenza- or adenovirus serotypes may be present.

An IgM antibody response is also absent in 20%–30% of influenza A and B virus infections (*Urquhart* 1974; *Buchner* et al. 1977; *Goldwater* et al. 1982). However, it is interesting that although the majority of the patients have previous influenza virus antibodies, the new influenza virus variants are in most cases antigenically distinct enough to stimulate the production of an IgM antibody response.

Other infections in which an IgM antibody response is not always elicited are those caused by echo- and coxsackieviruses (*Minor* et al. 1979; *Reiner* and *Wecker* 1981) and rotavirus gastroenteritis, although in the latter case the reports are somewhat contradictory, since some research groups have reported IgM antibody responses to occur in only 40%–60% of cases (*Sarkkinen* et al. 1979; *McLean* et al. 1980), whereas others have detected IgM antibodies in practically all cases (*Ørstavik* and *Haug* 1976; *Yolken* et al. 1978).

In reinfections the IgM antibody response is commonly missing, and in rubella the absence of an IgM antibody response has been used as indication of a reinfection and thus one probably not capable of causing congenital infections (*Boue* et al. 1971). However, IgM antibody responses have been observed in up to 20% of vaccinated persons challenged with live rubella virus vaccine (*Harcourt* et al. 1980). Thus, these results show that primary and secondary rubella virus infections cannot be reliably differentiated by the IgM antibody response.

In addition to immunity caused by natural infection and vaccination with live vaccines, inactivated vaccines also seem to be able to modify the immune response to a subsequent "secondary" infection and cause suppression of the IgM antibody response (*Ukkonen* and *Penttinen* 1981).

In reactivations of latent infections the IgM antibody response may be present or absent. In reactivations of herpes simplex virus the IgM antibody response has been observed only rarely and then mostly in cases with severe secondary infections such as herpesvirus encephalitis (*Kalimo* et al. 1978; *Van Loon* et al. 1981b). In contrast, an IgM antibody response has been detected in the majority (70%–80%) of cases with herpes zoster (*Cradock-Watson* et al. 1979a; *Arvin* and *Koropchak* 1980; *Tedder* et al. 1981). Regarding the appearance of IgM antibody responses in cytomegalovirus reactivations, variable figures have been published, from 7% (*Kangro* 1980) and 32% (*Van Loon* et al. 1981a) to 78% (*Cappel* et al. 1978).

The number of cases in which IgM antibodies can be detected is greatly dependent on the sensitivity of the assay used, and with the development of more sensitive assays in future, the number of cases found to be missing an IgM response may decrease. Still, it is evident that there are situations in which a recent infection cannot be excluded by a negative IgM antibody result and in which other tests will provide a more sensitive and reliable way toward a specific etiological diagnosis.

3.2.3 Variations in the IgM Antibody Response

A third problem in the interpretation of IgM antibody results is the temporal variation in the IgM response. IgM antibodies usually appear early during illness, but when heterogeneous populations are studied considerable individual variation can be observed. Table 3 shows the appearance of rubella virus IgG and IgM antibodies in the routine diagnostic material of 490 rubella infections diagnosed in our laboratory during the 3-year period from 1978 to 1981. Although rubella virus infection could be diagnosed in 45% of the cases from specimens taken 1 day after the onset of rash, a negative rubella virus antibody result on the 4th day did not exclude an acute rubella infection. Therefore, negative IgM antibody results at an early stage of a disease must be interpreted carefully. In other infections much longer delays in the appearance of antibodies have been observed, thus, e.g., in Argentine hemorrhagic fever IgM anti-

Table 3. Appearance of IgG and IgM antibodies in rubella virus infection in the routine diagnostic material of 490 rubella cases

Immuno-globulin class	Antibody-positive specimens (%) Days after onset of rash						
	0	1	2	3	4	5	6
IgM	12	45	62	85	93	100	100
IgG	7	14	38	64	76	96	100

Diagnosis was carried out at the Department of Virology, University of Turku, during the 3-year period 1978–1981. The antibodies were detected by indirect solid-phase RIA

bodies to Junin virus appeared as late as 14 days after the onset of symptoms (*Cossio* et al. 1979).

Another temporal variation is the prolonged IgM antibody response, which makes the timing of the infection difficult to establish on the basis of IgM antibody titers. Prolonged IgM antibody titers have been observed in complicated infections, e.g., rubella complicated by carpal tunnel syndrome, arthritis, or thrombocytopenic purpura (*Haire* and *Hadden* 1970; *Robertson* and *Bell* 1974), post-measles encephalitis (*Vuorimaa* et al. 1978), and severe cases of Japanese encephalitis (*Edelman* et al. 1976; *Burke* and *Nisalak* 1982). Prolonged rubella virus IgM responses have been observed in women who have contracted the disease in early pregnancy and given birth to a congenitally infected child (*Baublis* and *Brown* 1968; *Desmyter* et al. 1971; *Stallman* et al. 1974). The longest antibody persistence reported has been more than 4 years (*Stallman* et al. 1974). Prolonged persistence of BK virus antibodies has also been connected with infections during pregnancy (*Gibson* et al. 1981). Prolonged persistence of IgM antibodies to cytomegalovirus (*Nagington* 1971; *Cappel* et al. 1978) and BK virus (*Jung* et al. 1975; *Flower* et al. 1977) are common in immunosuppressed patients who become infected or in whom infection is reactivated after renal transplantation. Occasionally, prolonged IgM antibody responses have also been observed without any special reason (*Monath* 1971; *Pattison* et al. 1975; *Leidel* et al. 1977; *Meurman* 1978).

Prolonged IgM antibody responses have been detected in chronic infections in which they are considered to indicate an active replication of the virus. Thus IgM anti-HB_cAg has been detected in about 90% of patients with chronic hepatitis (*Gerlich* et al. 1980; *Roggendorf* et al. 1981a) but also in many "healthy" HB_sAg carriers (*Roggendorf* et al. 1981a; *Lemon* et al. 1981), even in those patients with normal liver histology, and for periods exceeding 4 years (*Dormeyer* et al. 1981).

When more sensitive methods are developed for the detection of specific IgM antibodies, the time after an infection during which they are detectable will be extended. For the diagnosis of an acute infection an ideal persistence of IgM antibodies would be 2–3 months, and therefore it is sometimes necessary to artifically decrease the assay sensitivity so that an optimal persistence time for the IgM antibody response is obtained. The easiest method applicable to most of the assays is to test the serum specimens at high dilution. Thus, in tick-borne encephalitis the persistence of IgM antibodies, as measured by a solid-phase anti-IgM EIA, was more than 6 months and

about 2 months when the sera were tested at dilutions of 1:1000 and 1:10000, respectively (*Roggendorf* et al. 1981b). Another method used in solid-phase anti-IgM assays is to dilute the test specimen with normal human serum, which then competes with the test specimen for the anti-IgM on the solid phase (*Decker* et al. 1981).

4 Conclusions

The value of the determination of specific IgM antibodies is variable depending on the virus and the infection in question. Constant and transient IgM responses seem to be a characteristic feature of infections which are caused by viruses with antigenic uniformity and which elicit a long-lasting immunity. Good examples of such infections are rubella (*Meurman* 1978; *Vejtorp* et al. 1979), measles (*Vuorimaa* et al. 1978), mumps (*Ukkonen* et al. 1981), infectious mononucleosis caused by Epstein-Barr virus (*Nikoskelainen* and *Hänninen* 1975), varicella (*Cradock-Watson* et al. 1979a), tick-borne encephalitis (*Heinz* et al. 1981), and hepatitis A (*Flehmig* et al. 1979). In all these, an acute infection can be reliably diagnosed or excluded by IgM antibody determination from a single serum specimen.

In infections caused by viruses which belong to a group of several closely related strains or serotypes, as is the case with enteroviruses, adenoviruses, parainfluenza viruses, and some togaviruses, the IgM antibody diagnosis is complicated by the possible absence of an IgM response as well as by possible false-positive reactions to related viruses. A missing IgM antibody response also complicates the diagnosis of reinfections and reactivations of latent viruses. However, also in these infections the serological diagnosis can often be improved by the determination of specific IgM antibodies.

The rapid development of immunological techniques during recent years, and the application of this modern knowledge to virus serology have created several sensitive and reliable methods for the determination of specific IgM antibodies. These methods are now available to practically every viral diagnostic laboratory, and when adopted for routine use they will bring considerable improvement to viral diagnostic services. The methods to be selected by individual laboratories depend, on the one hand, on the facilities and experience available, and on the other hand, on the daily or weekly number of specimens requiring testing for IgM antibodies. For small laboratories with limited facilities and a small number of specimens, methods based on serum fractionation may still be the most practical. However, for large-scale routine use the new solid-phase immunoassays, which offer good possibilities for automation and standardization, are clearly the methods of choice. The possibility of using commercial reagents and the increasing supply of prepackaged kits will in the future further increase the availability of these assays.

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Detection of Viral Antigens by Time-Resolved Fluoroimmunoassay

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1 Introduction

Highly sensitive immunoassays have proved to be valuable in the diagnosis of viral infections because viral antigens can be directly detected in clinical specimens by these assays. This technology was first widely used in the radioimmunoassay of hepatitis B surface antigen in serum (*Ling* and *Overby* 1972) and later applied to the detection of gastroenteritis viruses in stool (*Halonen* and *Meurman* 1982). Our group has also developed assays for the detection of seven respiratory viruses in nasopharyngeal secretions (*Sarkkinen* et al. 1981a–c), and these assays have now been successfully applied on a routine basis in our diagnostic laboratory. Immunoassays are now widely used in the detection of antiviral antibodies and particularly in the assay of IgM antibodies.

The immunoassays mainly used in the detection of viral antigens and antiviral antibodies are radioimmunoassays (RIAs) and enzyme immunoassays (EIAs). The advantages and disadvantages of RIAs and EIAs are well known. In brief, RIAs are

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highly sensitive, they can be well standardized and semiautomated, and the results can be printed in analyzed form by computerized detectors. However, problems are caused by the radiation hazard, the counting time of at least 1 min, and the relatively short half-life of the isotopes (e.g., [^{125}I]) used as labels. EIAs usually have the same sensitivity as RIAs if identical immunoreagents are used in both assays (*Sarkkinen* et al. 1980), they cause no radiation problems, the conjugates are stable, and the measurement time of the color reaction is short. Here, problems are caused by the need for an additional reaction with substrate requiring a further incubation step and washing, and by the difficulty, of standardization.

For these reasons, nonisotopic immunoassays in which the probe can be directly detected are highly desirable. One of the candidates for such an assay has been the fluoroimmunoassay, but background fluorescence caused by biological and other materials has decreased the sensitivity of conventional fluoroimmunoassays. A new possibility for using fluorescence in immunoassays is the application of the time-resolved fluorescence principle (*Soini* and *Hemmilä* 1979), and preliminary experience indicates that time-resolved fluoroimmunoassays (TR-FIA) can be applied to the detection of viral antigens (*H. Siitari* et al., Detection of HBsAG using time-resolved fluoroimmunoassay, in preparation) and antiviral antibodies (*Meurman* et al., to be published), as well as hormones (*K. Pettersson* et al., Time resolved fluoroimmunoassay of human chorionic gonadotropin – A new generation of nonisotopic immunoassays, in preparation). This review summarizes our experience in using TR-FIA in the detection in clinical specimens of viruses causing gastroenteritis and respiratory illness.

2 Principles

The principle of time-resolved fluorescence is shown in Fig. 1 (*Soini* and *Hemmilä* 1979). A fluorescent probe with a long lifetime is excited by a short light pulse, and specific fluorescence is measured after a selected delay time. During the delay time, background fluorescence which has a short decay time is eliminated and specific fluorescence is detected with a similar or higher sensitivity than that obtained with RIAs or EIAs. Lanthanides, such as europium (Eu), typically have a long decay time of, e.g., 100–1000 μs. An additional advantage of lanthanide chelates is that there is a large difference (Stoke's shift) between the excitation and emission wavelengths, which results in further reduction in background noise and improves the possibility of detecting specific fluorescence. Labeling antibodies with Eu can be done using ethylenediaminetetraacetate (EDTA), which binds efficiently to lanthanides. An aminophenyl derivative of EDTA has been synthesized which can be linked to proteins by the standard coupling reactions used in protein chemistry.

The principles of solid-phase indirect TR-FIA are the same as those of other indirect immunoassays. The technique used for detection of viral antigens is shown in Fig. 2. Polystyrene beads are used as the solid phase. As primary capturing antibody in the solid phase, the total immunoglobulin (Ig) fraction of guinea pig anti-virus hyperimmune serum is employed. The secondary antibody is the Ig fraction of rabbit anti-virus hyperimmune serum, and the indicator antibody is immunosorbent-purified anti-rabbit IgG antibody labelled with the Eu chelate.

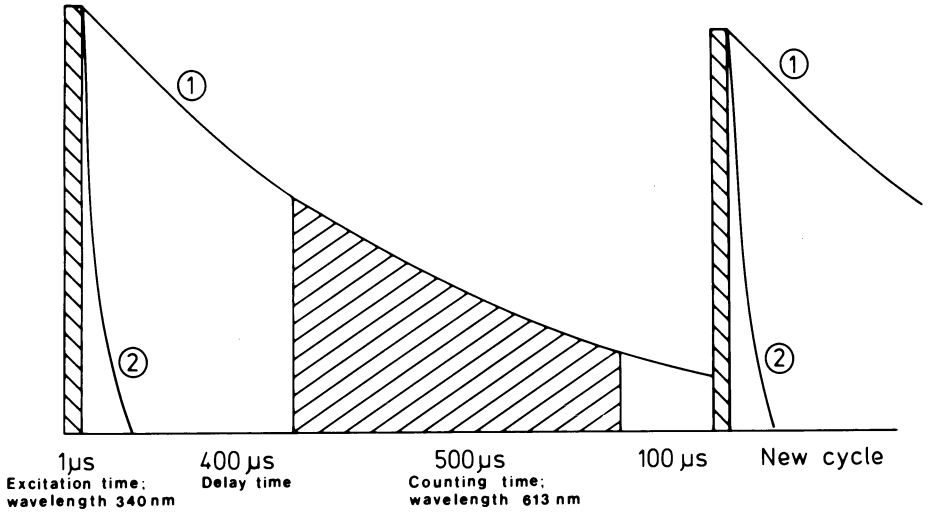

Fig. 1. Detection principle of time-resolved fluorescence. *1* Fluorescence of Eu chelate; *2* Short-decay background fluorescence (actual decay time may be less than 1 µs)

Layer	Immunoreagent Type	Immunoreagent Concentration	Incubation at 37 °C
Eu-chelate-labeled anti-rabbit IgG antibody	Immunosorbent-purified antibody	10-100ng/200µl	1 hour
Rabbit anti-virus antibody	Ig fraction	0.8-3.2 µg/200µl	1 hour
Specimen	Virus	1ng → 1mg/ml	1 hour (stool)
Guinea pig anti-virus antibody	Ig fraction	0.25-1.0µg/bead	16 hours (nps)
Polystyrene bead			

Fig. 2. Principle of the indirect time-resolved fluoroimmunoassay for the detection of rotavirus and adenovirus in stool and RSV, parainfluenzavirus types 1, 2, and 3, influenza virus types A and B, and adenovirus in aspirates of nasopharyngeal secretions (*nps*)

3 Methods

The indirect solid-phase TR-FIA used in the detection of viral antigens was selected because identical RIA procedures are in routine use in our diagnostic laboratory for the detection of rotavirus and adenovirus in stool and for the detection of seven respiratory viruses in nasopharyngeal secretions. For this reason, well-standardized RIAs are available as reference tests. Direct TR-FIAs are also being developed, and preliminary results indicate that they can be worked out in the same way as direct RIAs and EIAs.

Table 1. Titration of optimal dilutions of Eu-chelate-labeled anti-rabbit IgG antibody for the assay of purified adenovirus hexon antigen

Hexon concentration (ng/ml)	Eu-chelate-labeled antibody (ng/ml)									
	100		50		25		12.5		6.25	
	cps[a]	Ratio[b]	cps	Ratio	cps	Ratio	cps	Ratio	cps	Ratio
100	56.8	7	45.3	15	23.6	11	17.3	17	3.9	6
10	24.2	3	12.0	4	7.3	4	5.6	6	3.2	5
1	12.6	1.6	5.8	1.9	2.8	1.3	1.9	1.9	1.1	1.8
0	7.9		3.1		2.1		1.0	1.0	0.6	

[a]cps antigen sample × 10^3; [b]cps of hexon/cps of diluent control

3.1 Eu Chelate Labels

Aminophenyl-EDTA-Eu was synthesized using a modification of the Sundberg method (*Sundberg* et al. 1974) and conjugated to antibody by the diazoreaction (*Leung* and *Meares* 1977). Immunosorbent-purified sheep anti-rabbit IgG antibody (*Halonen* et al. 1980) was used in the conjugation, and the optimum degree of labeling was 3–5 Eu atoms/IgG molecule. Optimal concentrations of the labeled antibodies were determined by pretitration, as indicated in Table 1, and a relatively wide range of concentrations, between 10 and 100 ng of labeled antibody/200 µl, can be used in the test.

In each test a control was included in which the appropriate dilution of labeled antibody was incubated with a bead coated with 2 µg rabbit IgG. The ratio of bound activity to total activity was 20%–40%, depending on the batch of label, whereas the same immunosorbent-purified antibody labeled with [^{125}I] gives a ratio of 5%–10%.

Labeled antibodies were stored in Tris-buffered saline, pH 7.7, containing 0.5% bovine serum albumin and 0.05% sodium azide. No inactivation of label activity has been observed after storage for more than 1 year at 4 °C or at –60 °C. The Eu chelate quantum yield has also proved stable.

3.2 Antiviral Antibodies

Guinea pig and rabbit hyperimmune sera were prepared by immunization with either purified adenovirus hexon antigen (*Halonen* et al. 1980), purified tissue-culture-grown Nebraska calf diarrhoea rotavirus (*Sarkkinen* 1981), or nucleocapsids mixed with other virus-specific proteins in the case of respiratory syncytial virus (RSV), influenzavirus A and B, and parainfluenzavirus, types 1, 2, and 3 (*Sarkkinen* et al. 1981a–c). Ig fractions were prepared by precipitation of hyperimmune sera with an 18% (w/v) solution of sodium sulfate, followed by chromatography on a Sephadex G-25 column. The optimal concentrations of guinea pig Ig-adsorbed polystyrene beads and rabbit Ig were determined to be 0.25–1.0 µg guinea pig Ig/bead and 0.8–3.2 µg rabbit Ig/200 µl. Higher sensitivities are obtained in assays where low concentrations of guinea pig Ig can be used.

3.3 Incubation Period

Incubation of the specimens with antibody-coated beads was for 1 h at 37 °C for stool specimens and overnight at 37 °C for nasopharyngeal specimens. These incubation times were chosen because the concentrations of rotavirus and adenovirus hexon antigen are usually very high in stool specimens (up to several hundred micrograms/gram stool) and because proteolytic enzymes in stool degrade the immunoreactants during longer incubation times resulting in lower specific binding values. In respiratory specimens virus concentrations are usually lower (no more than 10 μg virus protein/ml nasopharyngeal mucus, and usually only a few hundred nanograms milliliter), and no decrease of specific binding values after longer incubation periods is found. Overnight incubation increases the sensitivity of the assay five- to tenfold compared with 1 h incubation.

3.4 Diluents

The diluents used in indirect TR-FIA are the same as those used in indirect RIAs. For specimens and rabbit Ig the diluent is phosphate-buffered saline, pH 7.3 (PBS), containing 20% inactivated fetal calf serum, 2% Tween 20, and 0.1% sodium azide. The label diluent is the same except that the fetal calf serum is replaced with sheep serum.

3.5 Specimens

From stool specimens, usually collected from hospitalized children with acute gastroenteritis, a 10% suspension in PBS is made and clarified by low-speed centrifugation. The supernatant fluid is diluted twofold in the TR-FIA diluent to give a final dilution of twenty-fold.

Nasopharyngeal specimens are usually collected from hospitalized patients with acute respiratory diseases. Specimens are collected with a disposable mucus extractor (Infant Mucus Extractor, Vygon, Ecouen, France) by aspirating secretions through the nostrils from the nasopharynx using the technique described by *Gardner* and *McQuillin* (1968). The specimens are sent to the virus laboratory without prior cooling. Storage for 1–2 days at room temperature does not significantly decrease the antigen titer. The mucus is diluted fivefold with the TR-FIA diluent and sonicated for 2 min using a Branson Sonifier Cell Disruptor B15 (Branson Instruments Co., Stanford, Connecticut, USA).

3.6 Procedures

Polystyrene beads (6.4 mm in diameter; Precision Plastic Ball Co., Chicago, Ill., USA) are coated with antiviral guinea pig Ig by incubating the beads overnight at room temperature in a solution containing 1.25–5.0 μg Ig/ml (0.25–1.0 μg/bead). Carbonate buffer, pH 9.6, is used as diluent for the antibodies. The beads are stored in the antibody solution at 4 °C until used, usually not for longer than 2 weeks.

Volumes of nasopharyngeal specimens (200 μl; fivefold dilution) or stool specimens (20-fold dilution) are pipetted into disponsable polystyrene tubes, and a polystyrene bead coated with antiviral Ig is then added. Both anti-adenovirus hexon and anti-rotavirus Ig-coated beads are always used in the tests with stool specimens, and at least three, but usually all seven of the anti-respiratory virus Ig-coated beads are used in the tests with nasopharyngeal secretions. After 1 h (stool) or overnight (nasopharyngeal secretions) incubation, the specimens are aspirated and the beads are washed with saline using a semiautomatic ten-channel washer (Dispenser-Aspirator DA-10, Pharmacia Diagnostic AB, Uppsala, Sweden). A 200-μl volume of rabbit antiviral Ig at optimal dilution (4–16 μg/ml) is then added to each tube, and the beads are incubated at 37 °C for 1 h. After the incubation step the beads are washed as described above, 200 μl Eu-chelate-labeled anti-rabbit IgG is then added to each tube, and the beads are incubated for an additional hour at 37 °C. After a final washing step the beads are placed in clean tubes and, after addition of 500 μl 15 μM 2-naphthoyltrifluoroacetone in aqueous detergent solution (counting solution), they are incubated for 15 min at room temperature and their fluorescence measured in a single-photon-counting fluorometer equipped with a Xenon flash lamp (*E. Soini* and *H. Kojola*, Time-resolved fluorometer for lanthamide chelates-new generation of non-isotopic immunoassays, in preparation). The excitation wavelength is 340 nm and the length of the excitation pulse is 1 μs. After a delay time of 400 μs single-photon emission is counted for 500 μs at 613 nm. After a further delay of 100 μs a new cycle begins. The cycle is repeated 1000 times during the overall counting time of 1 s. Delay and counting times can be admusted in the fluorometer, and several times other than those indicated above have been used without significant effect on the final results.

3.7 Expression of Results

The results are expressed as counts per second (cps) values. In each test the background fluorescence of the counting solution is measured in three tubes, and the mean value is deducted from the cps value of each tube. This background fluorescence value has been 2000–2500 cps in most of the experiments reported in this review, but more recently we have been able to reduce it to 400–600 cps after adjustments were made in the fluorometer. A label control using a bead preincubated in 200 μl of 10 μl/ml rabbit IgG is included in each test and the fluorescence value of this has been between 100 000–400 000 cps.

3.8 RIA and IF Procedures

Radioimmunoassay procedures are identical to those used in TR-FIA except that the anti-rabbit IgG antibody is labeled with [^{125}I]. Details of the RIA procedure have been reported elsewhere (Halonen et al. 1980). For immunofluorescence (IF), the standard indirect fluorescence method according to *Gardner* and *McQuillin* (1974) and commercially available reagents (Wellcome Research Laboratories, Beckenham, England) have been used.

4 Comparison of TR-FIA and RIA in the Detection of Rotavirus and Adenovirus in Stool

A comparison of TR-FIA and RIA in the assay of the reference hexon antigen is shown in Table 2. The sensitivities of TR-FIA and RIA are almost identical but the TR-FIA ratios rise with higher hexon concentrations, indicating a more linear dose response. Analysis of the precision profiles (*Ekins* 1981) shows similar standard deviations in each assay. This finding is to be expected since the primary capture Ig and the secondary Ig are identical in each assay. The "biological activity" of Eu-labeled antibody is higher than that of the iodinated antibody, since 42% of the activity was bound in the label control of the TR-FIA and only 6.3% in that of the RIA.

Table 2. Comparison of TR-FIA and RIA in the assay of purified adenovirus hexon antigen and the precision profiles of the assays

Hexon concentration (ng/ml)	TR-FIA			RIA		
	cps ± SD	SD		cpm ± SD	SD	
	($\times 10^3$)	ng/ml	%		ng/ml	%
100	28.9[a] ± 2.5	29.6	30	3536[a] ± 254	15.9	16
30	15.8 ± 1.7	4.8	16	1977 ± 153	6.1	20
10	7.3 ± 0.9	1.0	16	1204 ± 145	1.9	19
3	3.2 ± 0.4	0.6	19	509 ± 54	0.5	15
1	1.5 ± 0.2	0.3	25	282 ± 34	0.3	26
0	0.8 ± 0.3			150 ± 17		
Label parameters:						
Total activity	1110.0			80000		
Label control	468.0			5000		
Percent bound	42			6.3		

[a]Mean of ten assays

Table 3. Comparison of TR-FIA and RIA in the assay of purified rotavirus reference antigen (NCDV)

NCDV (ng/ml)	TR-FIA		RIA	
	cps ($\times 10^3$)	Ratio[a]	cpm	Ratio
100	53.8[b]	13	1393[b]	13
10	25.3	6	713	7
1	6.9	2	225	2
0	4.1		104	

[a] cps or cpm of NCDV sample/cps or cpm of diluent control; [b] Mean of three assays

Table 4. Comparison of TR-FIA and RIA in the detection of rotavirus and adenovirus in stool specimens collected from hospitalized children with acute gastroenteritis

Specimen		TR-FIA		RIA	
		cps ($\times 10^3$)	Ratio[a]	cpm	Ratio
Rotavirus negative		2.9[b]		81[b]	
Rotavirus positive no.	1	132.4	46	1130	14
	2	256.2	88	1600	20
	3	327.6	113	1454	18
	4	60.0	21	905	11
	5	477.2	165	1965	24
Adenovirus negative		4.9[b]		134[b]	
Adenovirus positive no.	1	180.2	37	2922	22
	2	51.1	10	651	5
	3	19.9	4	1107	8
	4	14.5	3	2593	20
	5	138.6	28	1032	8

[a]cps or cpm of virus sample/mean cps or cpm of negative specimens; [b]Mean of 10 negative specimens

Table 5. Results of a representative TR-FIA test in the detection of rotavirus and adenovirus in stool specimens

Controls and specimens		Control	Rotavirus	Adenovirus	Conclusion
Counting solution		2.5			
(mean value		2.5			
deducted)		2.5			
Label control		94.4			
Reference antigen					
	100 ng/ml		26.7	46.7	
	10		14.1	6.8	
	1		2.3	1.8	
	0		0.7	1.0	
Specimen no.	1		2.0	1.2	Negative
	2		62.3	1.3	Rotavirus +
	3		1.0	0.8	Negative
	4		0.9	42.2	Adenovirus +
	5		1.0	0.8	Negative
	6		0.7	0.6	Negative
	7		74.9	1.2	Rotavirus +
	8		44.9	1.5	Rotavirus +
	9		0.9	0.9	Negative
	10		0.9	1.0	Negative

In the rotavirus TR-FIA and RIA assays, the results are similar to those obtained for adenovirus (Table 3), but stool specimens positive for rotavirus show higher ratios in TR-FIA than in RIA when the ratios are calculated on the basis of the mean values for negative stools (Table 4). In the stools positive for adenovirus this higher ratio in TR-FIA is not consistently found.

The higher ratio of rotavirus TR-FIA has only a marginal effect on the percentage of positive stool specimens. Of 193 stool specimens tested simultanously, 29.5% were rotavirus positive using TR-FIA compared with 28.5% using RIA; the agreement between the two assays was 99.0%. In the adenovirus assays, positive specimens numbered 35.6% using TR-FIA compared with 35.2% using RIA; the agreement between the two assays was 98.1%. Typical test results of TR-FIAs in the detection of rotavirus and adenovirus in stool specimens are shown in Table 5.

5 Comparison of TR-FIA, RIA, and IF in the Detection of Respiratory Viruses in Nasopharyngeal Secretions

Comparisons of TR-FIA and RIA in the titration of the respiratory virus reference antigens (influenza virus A and B, parainfluenza virus, types 1, 2, and 3, RSV, and adenovirus) indicate similar sensitivities for these two assay procedures, but again TR-FIA ratios are usually greater with higher concentrations of the reference an-

Table 6. Comparison of TR-FIA and RIA in the assay of seven respiratory virus reference antigens

Reference antigen	Concentration of reference antigen (ng/ml)							
	100		10		1		0	
	TR-FIA cps × 10^3	RIA cpm	TR-FIA cps × 10^3	RIA cpm	TR-FIA cps × 10^3	RIA cpm	TR-FIA cps × 10^3	RIA cpm
Influenza A	42.9 (48)[a]	3127 (23)	6.8 (8)	1073 (8)	1.5 (2)	283 (2)	0.9	138
Influenza B	26.9 (38)	2044 (14)	5.3 (8)	660 (5)	1.1 (2)	200 (1)	0.7	142
Parainfluenza 1	34.7 (32)	1642 (10)	5.9 (15)	382 (2)	0.9 (2)	142 (1)	0.4	160
Parainfluenza 2	11.5 (29)	561 (6)	1.1 (3)	110 (1)	0.7 (2)	74 (1)	0.4	93
Parainfluenza 3	16.8 (7)	1361 (8)	3.3 (1)	323 (2)	1.9 (1)	189 (1)	2.5	169
RSV	2.7 (2)	361 (1)	1.3 (1)	290 (1)	1.1 (1)	270 (1)	1.3	262
Adenovirus	49.3 (82)	3220 (25)	12.5 (21)	1006 (8)	1.9 (3)	328 (2.5)	0.6	130

[a]Ratio of cps or cpm of virus sample/cps or cpm of diluent control

Table 7. Comparison of TR-FIA and RIA in the detection of RSV in sonicated aspirates of nasopharyngeal secretions collected from hospitalized children with acute respiratory infection

Specimen		TR-FIA		RIA	
		cps (mean) ($\times 10^3$)	Ratio[a]	cpm (mean)	Ratio
RSV negative no.	1	1.7		332	
	2	1.3		204	
	3	1.3 (1.3)		250 (278)	
	4	1.2		337	
	5	1.3		279	
	6	1.2		265	
RSV positive no.	7	22.3	17	2119	8
	8	4.5	3	493	1.8
	9	18.3	14	1945	7
	10	2.9	2	472	1.7
	11	16.4	13	1093	4

[a]cps or cpm of RSV sample/mean cps or cpm of negative specimens

Table 8. Number of positive specimens by RIA and TR-FIA in the detection of RSV, parainfluenza virus type 1 (Para-1) and adenovirus in sonicated aspirates of nasopharyngeal secretions collected from hospitalized children with acute respiratory disease

RIA	No. of specimens		TR-FIA		
			Positive		Negative
RSV positive	86		84		2
RSV negative	202		4		198
		Number of positive specimens:	RIA	86/288	(30%)
			TR-FIA	88/288	(31%)
		Agreement:	97.9%		
Para-1 positive	3		3		0
Para-1 negative	253		1		252
		Number of positive specimens:	RIA	3/256	(1.2%)
			TR-FIA	4/256	(1.6%)
		Agreement:	99.6%		
Adenovirus positive	8		8		0
Adenovirus negative	197		0		197
		Number of positive specimens:	RIA	8/205	(4%)
			TR-FIA	8/205	(4%)
		Agreement:	100%		

tigens (Table 6). In sonicated nasopharyngeal specimens the ratio of the TR-FIA cps value of the positive specimens to the mean cps value of the negative specimens is usually higher than the corresponding cpm ratio in RIA (Table 7). However, this slightly higher ratio does not significantly increase the number of positive samples (Table 8).

When TR-FIA results were compared with RIA and IF results in the detection of RSV in the nasopharyngeal secretions of 150 hospitalized children with acute respiratory disease, the agreement between the three tests was 93% (Table 9). This is an important finding since the immunoreagents for the IF test are completely different from those used in TR-FIA and RIA. For the detection of adenovirus in nasopharyngeal secretion the agreement between the three tests was 99%. The results of a representative TR-FIA test for the detection of three respiratory viruses (RSV, parainfluenza virus type 1, and adenovirus) in nasopharyngeal secretions of hospitalized children with acute respiratory disease are shown in Table 10.

6 Concluding Remarks

Using the fluorometer described in this review the detection sensitivity for the Eu chelate is 2–3 times higher than that for [^{125}I], according to theoretical calculations (*E. Soini* and *H. Kojola*, Time-resolved fluorometer for lanthanide chelates – new generation of non-isotopic immunoassays, in preparation), assuming a 1-s counting time for the fluorescent probe and a 1-min counting time for the [^{125}I]. This results from the superior statistical precision of fluorescent counting, the count rate being 100–1000 times higher than that of gamma counting. However, when the time-resolved fluorescence principle is used in the indirect immunoassay, the detection limit depends largely on the background values, which are determined by the immunological reactions between the primary capturing antibody on the solid phase, the secondary rabbit antibody, and the labeled indicator antibody. In this complex immunoassay system the sensitivity and precision of the TR-FIA procedure is the same or only marginally better than that of the identical RIA procedure, as presented in this review. The theoretically higher sensitivity of TR-FIA may be better observed in direct assays in which the antiviral antibody is labeled, and the background values caused by im-

Table 9. Comparison of IF, RIA and TR-FIA in the detection of RSV in aspirates of nasopharyngeal secretions collected from 150 hospitalized children with acute respiratory diseases

IF	RIA	TR-FIA	No. of specimens
+	+	+	56
–	+	+	5
+	–	–	4
–	+	–	1
–	–	+	1
–	–	–	83
Agreement	139/150 (93%)		

Table 10. Results of a representative TR-FIA test for the detection of RSV, parainfluenza virus type 1 (Para-1) and adenovirus in sonicated aspirates of nasopharyngeal secretions collected from hospitalized children with acute respiratory disease

Controls and specimens	Control	RSV	Para-1	Adenovirus	Conclusion
Enhancement solution	2.3[a]				
(mean deducted)	2.3				
	2.4				
Label control	64.4				
Reference antigen					
1000 ng/ml		11.5	44.7		
100		2.4	21.7	36.5	
10		1.7	3.4	11.5	
1		1.0	0.9	2.4	
0		0.7	0.4	0.6	
Specimen no. 1		1.8	0.7	1.3	Negative
2		20.5	0.6	0.9	RSV
3		1.7	0.4	1.1	Negative
4		25.0	0.7	1.4	RSV
5		26.3	0.6	0.9	RSV
6		1.5	12.5	0.9	Para-1
7		0.8	0.6	27.4	Adenovirus
8		1.0	0.5	0.6	Negative
9		1.6	0.6	0.9	Negative
10		25.8	0.6	25.4	RSV + adeno-virus

[a]Counts per second $\times 10^3$

munological cross-reactions are therefore lower. The most ideal assay system for time-resolved fluorescence may be a one-step method in which monoclonal antibodies against one determinant on the antigen are bound to the solid phase and monoclonal antibodies directed against another determinant on the same antigen serve as the labeled antibodies (*Uotila* et al. 1981). In this assay principle, the specimen and the labeled antibodies are incubated simultaneously, and the background values should be low. A one-step TR-FIA using the same antibodies in the solid phase as well as for the label has produced promising results (*H. Siitari* et al., Detection of HBsAG using time-resolved fluoroimmunoassay, in preparation).

Even assuming equal sensitivity, TR-FIA has significant advantages compared with RIAs and EIAs. In fact, it combines the advantages of both test methods. The major improvement of TR-FIA compared with RIA is the nontoxicity of the label. Eu chelates present no known biological hazards, and when conjugated with antibody, the antibody has retained its biological activity for more than one year and the Eu-chelate quantum yield has also been stable. Moreover, standardization of the tests can be carried out more precisely using stable labels, and conjugation methods previously used for enzyme and fluorochrome labeling can be directly applied to Eu chelates.

The counting time of 1 s is also an advantage of TR-FIA compared with RIA, since automation of the fluorometer may be less important when a short counting time is used.

The main advantage of TR-FIAs compared with EIAs is the fact that in EIAs an enzyme reaction catalyzing the turnover of a substrate is involved. This causes further difficulties in standardizing EIA tests, and the additional incubation and washing step required has also made EIAs less attractive in diagnostic laboratories where facilities are available for both assays. Unfortunately, no direct comparisons of TR-FIA and EIA results are available. However, when RIA and EIA were compared in our laboratory (*Sarkkinen* et al. 1979) no significant differences were found in the sensitivities of the two assay methods.

In addition to the detection of viral antigens, TR-FIA has also been succesfully applied to the assay of antiviral antibodies using the rubella virus IgG assay as a model (*Meurman* et al., to be published).

The experience reported in this review indicates that the TR-FIA technique offers clear advantages compared with RIA and EIA in the detection of viral antigens in clinical specimens. The number of positive specimens detected may not be significantly increased by using TR-FIA, but the availability of stable nonisotopic immunoreagents for a highly standardized test which is simple to perform will be of great benefit to diagnostic virus laboratories.

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Uses and Abuses of Diagnostic Electron Microscopy

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1 Introduction

It is almost exactly 20 years since it was realised that the electron microscopy technique of negative staining (*Brenner* and *Horne* 1959) could be applied in a practical fashion to the recognition of viruses not only in purified suspension, but also in association with cell material (*Almeida* and *Howatson* 1963). At first the technique was applied to the visualisation of viruses and the determination of their substructure. Very shortly afterwards it was realised that the technique could be applied to clinical material either directly or after one cycle of growth, either in egg or in tissue culture. At this time smallpox was still extant, and by far the most important use of the method was the differentation between variola and varicella virus (*Peters* et al. 1962; *Nagington* 1964).

During the subsequent years clinical virology in general and electron microscopy in particular progressed so that situations other than those dealing with immediate questions of safety were examined. The electron microscopy technique of negative staining became a tool in clinical virology, the use of which lead to the recognition of several new human and animal pathogens (*Tyrrell* and *Almeida* 1967; *Feinstone* et al. 1973; *Flewett* et al. 1973). This aspect of negative staining continues up to the present time, with more new viruses of clinical interest or significance being characterised by visualisation. In addition to this innovative work, the EM has also taken on the role of routine screening when other, less work intensive methods are not available.

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These various aspects of negative staining will be discussed, and the advantages and disadvantages of electron microscopy as compared with the more recent methods of radioimmunoassay and enzyme assay will be considered.

2 Materials and Methods

Almost any clinical specimen can be used for electron microscopy, although some make better starting material than others. For example blood, urine and faeces, with very little preparatory work, form suspensions that make suitable EM specimens. Solid tissues have to be made into homogenates, and although they have high levels of cellular debris they nevertheless make good EM material. Vesicular fluid needs only to be diluted in distilled water to provide a specimen that allows the detection of viruses such as herpes simplex. On the other hand, material with a high mucus content, such as nasopharyngeal secretions, are difficult to handle and do not yield good micrographs. Such specimens are best examined by immunofluorescence methods (*Gardner* and *McQuillin* 1980).

2.1 Methods of Negative Staining

A detailed account of specimen preparation and the parameters involved for successful negative staining has been published recently, and will not be discussed here (*Almeida* 1980). However, it is worth noting that the virus-containing material can be examined directly by placing a drop of the specimen on the grid, washing with distilled water, then replacing this with negative stain, most frequently phosphotungstic acid. Alternatively, the specimen is concentrated by centrifugation and the resulting pellet resuspended in a very small amount of distilled water. A drop of this suspension is then mixed with the contrast agent and the fluid placed on a microscope grid, excess fluid is withdrawn, and the grid examined in the microscope.

The advantages of the first method are speed, a matter of minutes, and the fact that it only requires approximately 5 μl of specimen. The second method increases the amount of virus in the specimen, because the virus is concentrated by centrifugation, and also allows virus to be separated from low molecular weight, proteinaceous material which is not spun down. By this method the virus appears more clearly than in the direct, on-the-grid method and consequently micrographs of higher resolution are obtained. One drawback of the second method is that it requires rather more starting material than the direct on-the-grid method. The usual amounts for centrifugation are in the 50–200 μl range, although more can be used. However, availability of starting material is a problem only in a minority of clinical specimens. Also, since there is a longer preparatory procedure, the overall time of preparation takes hours rather than the minutes required for the direct method. In spite of this, whichever method is employed, electron microscopy remains one of the fastest techniques available for obtaining a viral diagnosis.

Many specimens to be examined for the presence of virus give better results when antibody which is specific to the known or suspected virus is added the technique of immune electron microscopy. At the practical level it differs from the centrifuga-

tion procedure only in that a suitable antiserum is added to the virus-containing fluid or suspension and this is allowed to react for a suitable length of time, which can be as short as half an hour. The suspension is centrifuged as before and virus appears in the form of immune complexes.

2.2 Results Obtained by Negative Staining

The negative staining technique works on the basis that virus particles, for the most part, are not penetrated by negative stain but are surrounded by it. Since the negative stain contains a heavy metal, e.g., the tungsten of phosphotungstic acid or the uranium of uranyl acetate, it is virtually opaque to the electron beam. A virus particle, which is electron-lucent, appears as a light area within the dark of the surrounding stain (Fig. 1). Since very few clinical specimens are purified, they are likely to contain an excess of structures that are not virus particles. All of these will also be negatively stained. However, there are two features associated with viruses that greatly increase the likelihood of recognising them in even the crudest clinical specimen. These are, first, the geometric construction associated with many viruses and second, the fact that virus particles are more rigid structures than cell debris and because of this retain a more three-dimensional configuration. This latter property means that viruses, because of their greater height, are surrounded by a thicker layer of contrast agent and thus appear as light structures surrounded by a darker zone than that surrounding other components of the specimen (Fig. 2).

While these factors are general to all virus-containing specimens, there are also factors associated with individual virus types and even with individual specimens. First, there is a relationship between virus particle size and the titre of the virus that can be recognised in the EM. A large distinctive poxvirus can be recognised at levels of 10^5 particles/ml or even less of original specimen, whereas an enterovirus or rubellavirus would probably need to be present in the order of 10^7 particles/ml, although the

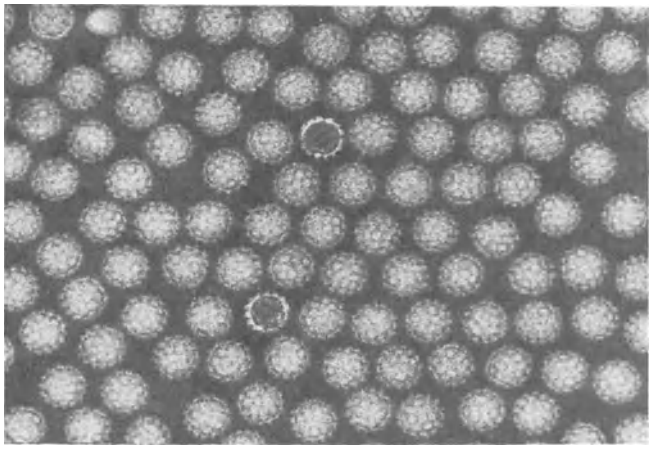

Fig. 1. A simple homogenate of a clinical wart specimen provided this micrograph. Electron-lucent wart virus particles appear light against the electron-opaque phosphotungstic acid background. × 130 000

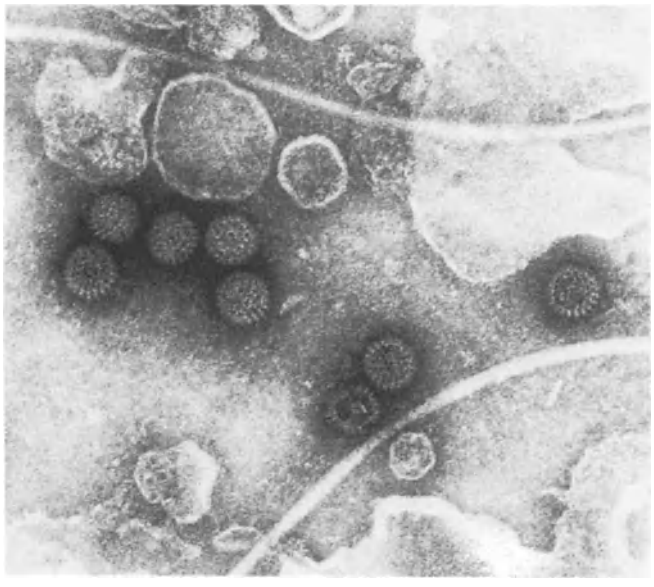

Fig. 2. Since virus particles are more three-dimensional than surrounding cell or bacterial debris, they have a more intense dark rim around them. In this micrograph there are numerous background structures but the rotavirus present appears prominent because of this phenomenon. × 130 000

reasons for the increased number required are different for the two viruses. In the case of the small 27 nm enterovirus, it does not displace as much stain as the 250 nm poxvirus and therefore does not have as much contrast against the background as the larger particle does. In the case of rubella virus, it is more difficult to recognise because the single particle looks very much like a fragment of cell material. However, in both these instances the sensitivity of the EM can be much increased by the use of immune electron microscopy. A real situation occurred recently when it was necessary to visualise yellow fever virus which, like rubella virus, is a member of the *Togaviridae*. Examination of the specimen without antiserum revealed occasional particles such as the one in Fig. 3a, which may or may not be a virus. Addition of antiserum revealed complexes such as the one in Fig. 3b which can be recognised with ease.

From the microscopist's viewpoint, those viruses that have a geometric construction are the easiest to visualise (*Almeida* and *Waterson* 1980). This group includes viruses with both cubic and helical symmetry. However, within the cubic virus group, those with distinct subunit construction, such as wart virus, rotavirus, and adenovirus, are better detected than the RNA cubic viruses such as the enteroviruses and rhinoviruses, which are small in size and have no visible subunit construction. Helical viruses such as the parainfluenza viruses and measles virus are frequently detected by the internal helical component alone, although once this has been found it is usually possible to locate at least some particles that also have a membrane component. Although not possessing geometric construction, poxviruses, by reason of their large size and distinctive construction can also be detected with ease even when there is a high level of background contamination such as one gets in suspensions that are derived from

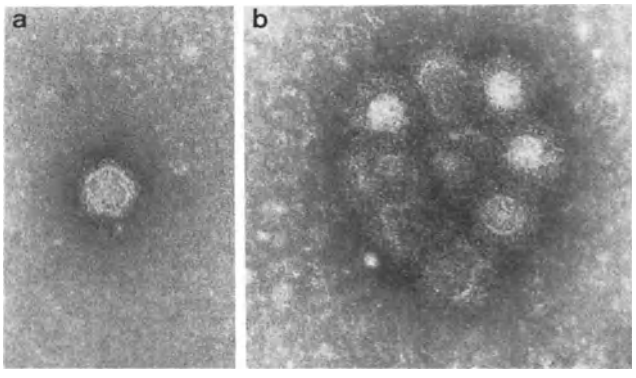

Fig. 3a. Direct examination of a specimen for yellow fever virus revealed only scattered particles that may or may not be virus. One of these particles is shown here. Although it displays a small surface fringe and has an angular outline, both viral characteristics, identity could never be proved. × 130 000. b) When specific yellow fever antiserum was added to the same specimen, complexes of antibody-covered particles were visualised. In this form, it is possible to identify the particles within the aggregate as yellow fever virus. × 130 000

scab material. Another group of viruses that have distinctive appearance but no geometric construction are the influenza viruses, which are recognised solely on their distinctive surface projections, the haemagglutinin and neuraminidase molecules of the virus. These viruses do have internal helical symmetry, but this is very rarely seen and is in no way necessary to allow a definitive diagnosis of influenza virus to be made (*Almeida* 1981).

Unfortunately, another virus group with distinctive surface projections, the coronaviruses, are more problematical to identify in the EM. On the one hand, they are frequently not recognised when they are present, and conversely, candidate coronavirus are put forward when none are present. Proof of non-recognition of coronavirus comes from a study carried out by the Central Public Health Laboratory, Colindale, London (*P.S. Gardner*, unpublished communication). A number of participating laboratories were sent specimens for examination by negative staining. The results showed that 92% of the laboratories correctly recognised herpesvirus and 88% detected rotavirus plus adenovirus. Those that did not get the correct results did not report the presence of a wrong virus, but simply did not detect virus at all. However, when a coronavirus was tested only 51% of the laboratories made a correct diagnosis, 33% found no virus at all and 16% reported a wrong virus in the specimen. It is therefore not surprising that examination of an actual clinical specimen frequently leads to a diagnosis of unknown coronavirus. The majority of these so-called coronaviruses are membrane fragments with a fringed surface. Unfortunately, endoplasmic reticulum, bacteria and mycoplasma, when broken down, yield fragments that assume spherical from and frequently display a fringe of surface projections (Fig. 4a). Similarly, the broken-down cristae of mitochondria can also appear in the virual size range and have most distinctive subunits on the surface (Fig. 4b).

However, it is possible to recognise coronaviruses in clinical specimens. Fig. 5 shows particles present in canine faeces, and these display the classic morphology that is displayed by tissue culture or egg-grown virus. It is also possible that some particles

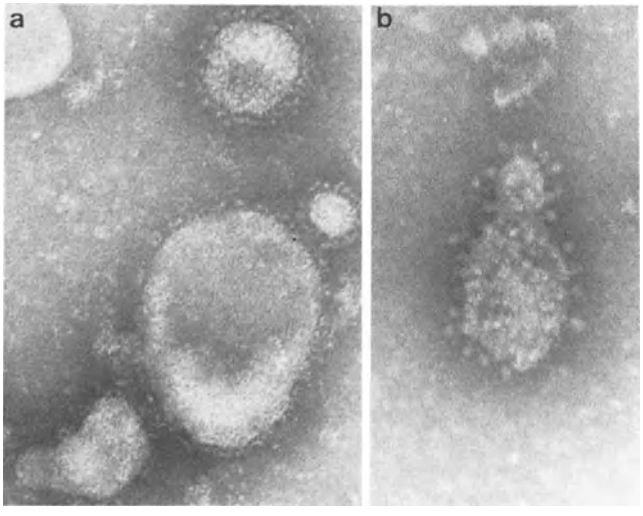

Fig. 4a. Approximately spherical, fringed particles can be found in almost all specimens examined by negative staining. It is important to recognise that these are not virus particles. The fringe is nondescript in nature and the particles are present in a wide size range. × 179 000. b) By negative staining, mitochondria show a distinct subunit structure that can be mistaken for virus. However, as can be seen on the mitochondrial fragment shown here the surface subunits are almost round whereas viral surface projections are usually elongated. × 175 000

with a morphology similar to but not identical to coronavirus may well belong to another group that has not yet been recognised. Until such particles have been further characterised it is as well to class as coronavirus only those biologically active particles having the classic morphology of leaf-shaped projections surrounding a lipoprotein membrane that frequently shows an umbilicate appearance (*Patterson* et al. 1975).

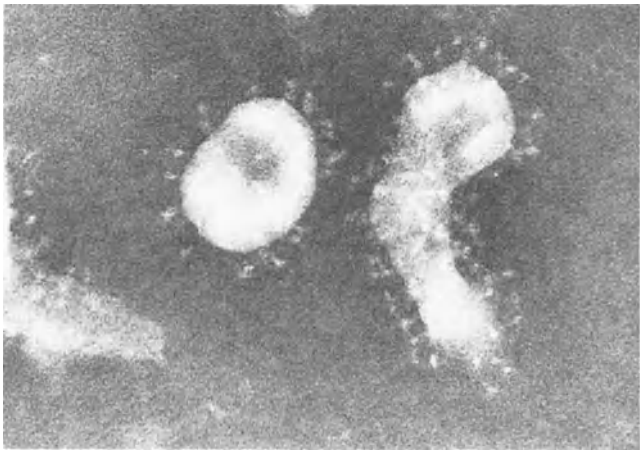

Fig. 5. Two typical coronavirus particles present in a specimen of canine faeces. Although pleomorphic, the particles have very distinctive projections on the surface and the particle on the *left* shows the umbilicate appearance associated with this virus group. × 7000

Very fortunately, the majority of specimens that come to the EM laboratory for viral diagnosis are likely to contain viruses that either have a geometric construction or one that is sufficiently distinctive to make recognition simple. Conversely, it may be that the successfully diagnosed specimens are those that contain readily detectable viruses, and this factor must be taken into consideration. For example, solid, warty skin lesions if positive for virus will contain either wart virus, orf virus or the poxvirus of molluscum contagiosum – all distinctive, easy-to-recognise viruses. Vesicular skin lesions will contain either a herpes- or a poxvirus, again viruses with a high recognition factor. Faecal samples will most probably be found to contain rotavirus, a most distinctive virus. The next most likely virus to be present is adenovirus, also a virus with a clear-cut morphology. Small cubic viruses are frequently found in faecal specimens and although these are less distinctive in appearance it is interesting how often they are detected in the form of clumps, which are probably naturally occurring immune complexes. Examination of serum is usually carried out for the detection of hepatitis B antigen, and although single particles, particularly of the small spherical type, might be difficult to recognise on their own, use of immune electron microscopy gives rise to complexes that are morphologically unmistakable (Fig. 6).

3 Discussion

3.1 Sensitivity

Numerous publications have compared the levels of sensitivity obtained by currently available diagnostic methods (*McIntosh* et al. 1980; *Obert* et al. 1981). Unfortunately, at this time there are very few established standard methods for carrying out any virus diagnostic technique, and variation between individual laboratories can be enormous.

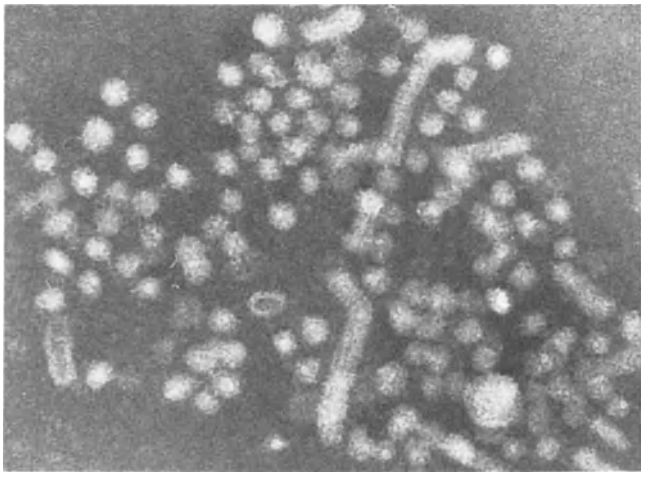

Fig. 6. On its own, the small spherical form of hepatitis B antigen (HBAg) is not easy to recognise. However, if antibody is added so that complexes are formed these are so distinctive that the sensitivity of the method equals that of enzyme-linked immunoassay. × 179 000

If one ignores biological recovery, which will always remain the most sensitive method of virus detection but which is not always feasible, and concentrates instead on those methods which rely on the presence of viral material, then radioimmunoassay is generally accepted as the most sensitive of available methods, with enzyme-linked immunoassay only somewhat behind. Electron microscopy is generally placed third on this sensitivity list, below the more modern radio-labelling and enzyme-labelling methods, but above the more conventional complement fixation and gel diffusion tests. However, a previous study showed that whereas one laboratory carrying out comparative tests on faeces by enzyme-linked immunoassay and electron microscopy found the enzyme assay was considerably more sensitive, a second laboratory examining the same specimens found that enzyme assay and electron microscopy were in total agreement and picked up exactly the same number of positives (*Almeida* 1981). In this context, it would appear that attention to detail in specimen preparation is all important for electron microscopy, and the extra work involved is minimal and often even less than has previously been carried out. For example, many workers centrifuge specimens for electron microscopy at forces of 40000*g* for several hours. Not only is this unnecessary, but the amount of low molecular weight protein deposited by this treatment can obscure any virus present.

At an absolute level, EM is capable of detecting 10^5-10^6 virus particles in the starting material. However, it must be remembered that the EM recognises not only biologically active virus but also non-viable virus as well as viral components, and in some instances this can mean detecting virus in a specimen with only 10^2 or 10^3 viable particles.

3.2 Positivity

Distinct from sensitivity is the ability of a method to obtain a positive result. If one considers a faecal specimen that has come into the laboratory for possible presence of virus, the most likely pathogen to be present in the specimen is rotavirus, and an enzyme assay for this virus is very likely to be carried out. If the result is negative it may be possible to examine the specimen again by enzyme assay for adenovirus. If this is negative it is unlikely at the moment that any other tests would be available in the clinical laboratory. However, if the same specimen is examined by electron microscopy, although the operator will look initially for rotavirus, if this is not found he will go on to look for any other candidate viruses in the specimen. In this context, electron microscopy is the most likely method to provide a positive result in specimens in which the type of virus implicated is unknown or only suspected. Alternatively, it can be said that electron microscopy is able to examine a specimen for the presence of virus in general rather than one virus in particular.

3.3 False Results

A negative finding for a specimen examined by electron microscopy must always be considered as a possible false negative. As stated, although the microscope may detect levels as low as 10^2 or 10^3 infectious particles it will not, in company with most tests based on the presence of viral components, detect levels of virus below this. Biologi-

cal recovery remains, theoretically as well as practically, the most sensitive method of detecting virus in a clinical specimen, since only one biologically active virus is needed for recovery. However, many specimens that do not contain detectable levels of virus can be accepted as true negatives on the basis that in order for the virus to be active in the disease it would have to be present at a level well above the threshold. A good example of this situation is the examination of diarrhoeal faeces for a causative virus. Low levels of virus are problematical, as it is difficult to assess whether or not they are implicated in the disease. It is therefore only those specimens that contain at least a moderate titre of virus that give a clear-cut picture and are acceptable positives.

False positive results theoretically should never be encountered in electron microscopy. Although a lipid-laden faecal specimen, or one with a high titre of protein A, might give a false positive result in an enzyme assay (*Yolken* and *Stopa* 1979), it will not do so when examined by negative staining. The virus itself must be visualised in order for the specimen to be scored as a positive. However, the word "theoretically" is important, as it is frequently possible for a careless operator to transmit virus from a positive to a negative specimen by means of the forceps used to hold the grid.

3.4 Application of EM Examination

This is the section to which the term "uses and abuses" the title of this review applies. Which samples are best examined by electron microscopy, which might be examined, and which should most definitely not be tested by this method? First, it is essential to distinguish between what can be described as research diagnosis and routine diagnosis.

Research specimens from conditions involving an uncharacterised virus or even from conditions in which a viral aetiology is only suspected should always be examined by negative staining. If possible, the specimens should be examined both in their original form and after the addition of antiserum. Obviously, a specimen from a condition with only a suspected viral origin is unlikely to have a ready-made antiserum, but a recovery serum from the same or a similar case can work very well. An example of this occurred when it was attempted to further characterise a parvovirus that had been found in association with aplastic crisis in sickle cell anaemia (*Serjeant* et al. 1981). Serum containing viral antigen was mixed with recovery serum and the resulting preparation revealed the presence of large viral complexes in the circulation of the patient with aplastic crisis. Although the virus had been previously characterised as a parvovirus on serological grounds, the EM study corroborated this finding by visualising typical parvovirus construction and also revealed the large number of virus particles implicated (Figs. 7a, b). Similarly, two recent studies employing immune electron microscopy have characterised the morphology of the particles associated with Korean haemorrhagic disease (*McCormick* et al. 1982; *White* et al. 1982). Since the number of specimens in the category described here is small, there is no problem in carrying out EM examination on all available material. Although many specimens may not give a positive result, the number that do will provide information that will facilitate further study.

Turning to routine diagnosis using electron microscopy, it is again possible to divide specimens into two categories, depending on whether or not a more suitable technique exists for the diagnosis of the virus implicated. Many viruses that are well

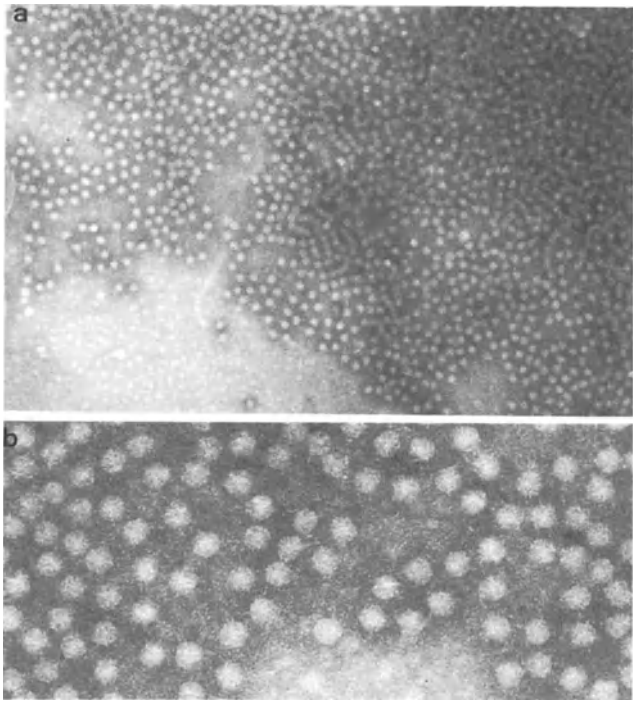

Fig. 7a. Examination of serum from a child with sickle cell anaemia during aplastic crisis revealed the presence of large numbers of a parvovirus. The specimen had been reacted with a recovery serum to make identification easier. The morphology of the particles visualised corroborated the serological finding that a parvovirus was implicated in this syndrome. × 49 500. b) A higher power micrograph of the same specimen showing virus structure in greater detail. × 130 000

characterised nevertheless form a very small proportion of the diagnostic load, and so it has not been found necessary to produce routine tests for them. A good example of this group are the viruses associated with warty skin lesions. The candidate viruses for this type of lesion are the human common wart virus, orf virus, and molluscum contagiosum virus, all well characterised, but the need to diagnose them occurs only infrequently and routine diagnostic tests are not available. In this situation by far the simplest approach is to make an homogenate of the wart and examine it by negative staining. This one test will recognise any of the three viruses that might be implicated. In situations of this type the EM is by far the most efficient means for arriving at a speedy diagnosis.

The last category of specimen is that of the routine specimen for which another, probably automated test already exists. Good examples of this specimen type are sera with hepatitis B antigen and faeces with rotavirus. A whole battery of tests now exists for the screening of sera and there are well-established enzyme immunoassays for rotavirus screening. Given this situation, the work-intensive electron microscopy becomes a very inefficient way of obtaining results even although the results will be accurate. In any large survey, often undertaken for epidemiological purposes, the highest level of sensitivity is not essential and it is much more important that large

numbers of specimens are examined. If, however, within such a survey a subset of specimens appears that gives anomalous results, these can with advantage be examined by negative staining.

Within this group are also those specimens which can be examined by electron microscopy but which are more likely to yield a positive result by another method. This applies particularly to specimens of nasopharyngeal secretion for the detection of respiratory viruses. As stated previously, these specimens are difficult to handle for electron microscopy and can be examined much more simply by the immunofluorescence method (*Gardner* and *McQuillin* 1980).

In summary, over the past 20 years or so the electron microscope has given information about virus morphology that has lead to the awareness of new virus types and the association of recognised virus groups with different clinical situations. The more recently developed techniques of enzyme immunoassay and radioassay have now taken over many of the more routine aspects of viral diagnosis, leaving the microscope to obtain diagnosis and information from the more unusual specimens and those for which, as yet, no standard method exists.

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The Use of Staphylococcal Protein A in Diagnostic Virology

DOUGLAS D. RICHMAN*

1 Introduction

Staphylococcal protein A (SPA), a protein component of the cell wall of *Staphylococcus aureus*, binds the Fc portion of many immunoglobulins of most mammalian species. *Verwey* first described SPA in 1940 as a protein antigen present in type A staphylococci (coagulase-positive, α-toxin-positive, mannitol-fermenting, and pathogenic; i.e., *S. aureus*), but not in type B staphylococci (those lacking these characteristics). The systematic study of antigen A did not begin in earnest until two decades later (*Jensen* et al. 1961; *Lofkvist* and *Sjoquist* 1963; *Grov* et al. 1964). Grov and associates proposed the designation "protein A" to avoid confusion with the antigenic polysaccharide A (*Grov* et al. 1964).

In 1966, *Forsgren* and *Sjoquist* demonstrated that the well-recognized ability of all human sera to agglutinate *S. aureus*, which had been attributed to the universal presence of so-called natural antibodies, did not in fact represent an immune reaction. They showed that purified SPA bound to the Fc fragment of the immunoglobulin molecule, not to the antigen-binding Fab fragment. This seminal observation that SPA was not merely an antigenic protein in the cell wall of *S. aureus*, but was an

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antibody-binding protein, catapulted SPA into prominence as a reagent for both immunochemistry and immunoassays (*Goding* 1978).

2 SPA in the Staphylococcus

Staphylococcal protein A was detected on 99% of the *S. aureus* strains examined in two studies (*Forsgren* 1970; *Lachica* et al. 1979) and in 90%–96% of those examined in three others (*Kronvall* et al. 1971; *Winblad* and *Ericson* 1973; *Bind* et al. 1978). Many of the SPA-negative strains observed in one study were methicillin-resistant organisms (*Winblad* and *Ericson* 1973). No strains of *S. epidermidis* (defined as both coagulase- and deoxyribonuclease-negative) or *S. hyicus* were found to contain SPA (*Forsgren* 1970; *Kronvall* et al. 1971; *Lachica* et al. 1979), although 4 of 127 animal isolates of coagulase-positive *S. intermedius* were described as SPA-positive (*Lachica* et al. 1979). There is marked variation in the amount of SPA in different strains. The Cowen I strain synthesizes relatively large amounts of SPA. This strain has been the prototype for the production of SPA and for the study of the molecule, which comprises 1.7% of the dry weight of whole, lyophilized cells and 6.7% of the dry weight of isolated cell walls (*Sjoquist* et al. 1972a). SPA is covalently linked to the cell wall peptidoglycan, from which it is released by lysostaphin digestion of cells (*Sjoquist* et al. 1972b). The SPA molecules are densely distributed on the surface of the bacterium, with approximately 80 000 molecules/cell as determined by Scatchard analysis (*Kronvall* et al. 1970b). Only a small fraction of the SPA is newly synthesized (cytoplasmic) or secreted extracellularly (*Forsgren* 1970; *Movitz* 1976). It is of interest that SPA is synthesized at the same rate but is totally secreted into the medium if the cells are grown as protoplasts in sucrose-stabilized medium containing lysostaphin (*Movitz* 1976).

The preparation for formalin-fixed, heat-killed whole *S. aureus* cells as immunoadsorbents is relatively simple (*Goding* 1978; *Kessler* 1981). The preparation of purified SPA from a lysostaphin digest of cells, or from a methicillin-resistant strain that produces only extracellular SPA, is readily accomplished by affinity chromatography on IgG-Sepharose (*Hjelm* et al. 1972; *Lindmark* et al. 1977). Killed whole cells are commercially available from several sources; highly active and pure SPA is also commercially available.

Although the biological role of SPA has not been defined, the potential advantage to the bacterium of immobilizing antibodies, by binding the Fc portion (which is recognized by phagocytic host cells) and thus directing the antigen-recognizing Fab portion away from the organism, appears obvious. In vitro studies have demonstrated that SPA suppresses both phagocytosis and chemotaxis by polymorphonuclear leukocytes (*Dossett* et al. 1969; *Verhoef* et al. 1979; *Colburn* et al. 1980; *Musher* et al. 1981). In vivo studies have not been able to show an advantage of SPA-containing strains over SPA-negative strains; however, these studies have all been carried out in murine models (*Forsgren* 1972; *Hsieh* et al. 1978; *Gross* et al. 1978). Because SPA binds 100-fold less immunoglobulin from murine serum than from human or rabbit serum (*Langone* 1978; *Richman* et al. 1982), the mouse may not be an appropriate model in which to examine the biological role of SPA.

3 Biochemical Characterization of SPA

Staphylococcal protein A is a highly stable 42 000 molecular weight protein which retains its activity following exposure to 4*M* urea, 4*M* thiocyanate, 6*M* guanidine hydrochloride, or pH or temperature extremes (*Sjoholm* 1975). It also retains its activity following conjugation to a number of molecules (Table 1). It has an extended, rather than a globular shape as determined by its frictional ratio of 2.1–2.2 and its intrinsic viscosity of 29 ml/g (*Sjoquist* et al. 1972b; *Bjork* et al. 1972), and it contains few if any sugar residues (*Sjoquist* et al. 1972b). Its isoelectric point is 5.1 (*Lindmark* et al. 1977). SPA contains α-helical regions that comprise about 50% of the molecule (*Sjoholm* 1975; *Deisenhofer* 1981). Analysis of tryptic digests reveals four highly homologous Fc-binding regions, each consisting of a nearly identical sequence of approximately 60 amino acids (*Sjodahl* 1977). These Fc-binding regions are arranged in tandem in the N-terminal portion of the protein. The C-terminal end of the molecule is bound to the bacterial cell wall and consists of the remaining 150 amino acids that are not associated with the repetitive Fc-binding sequences (*Sjodahl* 1977). The contact domain of SPA with immunoglobulin, as determined by crystallography, is hydrophobic (*Deisenhofer* 1981).

Many of the amino acids in SPA, including tyrosine, are present in multiples of four, which is consistent with the presence of four highly homologous repetitive sequences (*Sjoquist* et al. 1972b; *Sjodahl* 1977). The presence of these multiple binding sites permits SPA to be iodinated without the total loss of its ability to bind immunoglobulin; nitration of all the tyrosyl residues with tetranitromethane abolishes the Fc binding (*Sjoholm* et al. 1973). The loss of reactivity of SPA following iodination is directly proportional to the number of tyrosyl residues that are iodinated (*Sjoholm* et al. 1973).

Table 1. SPA conjugates that have been used in immunoassays

SPA conjugate	Published example of use
Erythrocytes	*Ghetie* et al. 1974b; *Nardiello* et al. 1982
[^{125}I]	*Dorval* et al. 1975; *Langone* 1978;
[^{3}H]	*Wilder* et al. 1979
Fluorescein	*Ghetie* et al. 1974a; *Biberfeld* et al. 1975; *Forsum* et al. 1976
Ferritin	*Bachi* et al. 1977
Gold	*Roth* et al. 1978; *Muller* and *Baigent* 1980; *Geuze* et al. 1981; *Slot* and *Geuze* 1981
Invertase	*Surolia* and *Pain* 1981
Glucose oxidase	*Pain* and *Surolia* 1979
Peroxidase	*Dubois-Dalcq* et al. 1977; *Surolia* and *Pain* 1979
Alkaline phosphatase	*Engvall* 1978; *Crowther* and *Abu Elzein* 1980
Biotin	Unpublished observations

The conjugates and examples listed are for illustrative purposes only and do not represent an exhaustive list of either conjugates or published examples

4 Reactivity of SPA with Immunoglobulins

Staphylococcal protein A reacts almost exclusively with the sera of mammals. Utilizing two qualitative assays of SPA reactivity with sera, *Kronvall* et al. (1970a) showed essentially no reactivity with the sera of fishes, amphibia, reptiles, and birds, with the single exception of a primitive flightless bird, *Rhea americana*. In contrast, SPA binds to serum immunoglobulins of almost all mammalian species (*Kronvall* et al. 1970a); however, there is a greater than 10^6-fold variation in the affinity of SPA for the immunoglobulins of different species (*Langone* 1978; *Richman* et al. 1982). The range in SPA-binding capacity of the sera from several laboratory and domestic animals is shown in Table 2. Although a wide interspecies variation in the SPA-binding capacity of serum immunoglobulins exists, only small variations occur among individuals within the same species, with one notable exception. A greater than 10^4-fold variation in SPA-binding capacity was observed among the sera of nine goats. Interspecies differences in quantitative serum SPA-binding capacity correlates well with taxonomic differences, and the serum SPA-binding capacities of many African mammals correspond closely to that of their laboratory relatives (*Richman* et al. 1982).

In both humans and animals there are marked differences in the affinity of different classes and subclasses of immunoglobulins for SPA. For example, human IgG1, IgG2, and IgG4 bind strongly. IgG3, which does not bind to SPA, comprises only 1%–3% of total human immunoglobulin. IgA2 and some IgM proteins also bind well. These determinations have been made by examining both whole normal sera and panels of myeloma proteins (*Kronvall* and *Williams* 1969; *Saltvedt* and *Harboe* 1976; *Patrick* et al. 1977). In addition, 6%–9% of IgE proteins bind to SPA, but probably in a manner different from that of the other immunoglobulins (*Johansson* and *Inganas*

Table 2. SPA-binding capacity of sera from laboratory and domestic animals *Richman* et al. (1982)

Species	Number of sera	SPA-binding capacity[a]	
		Geometric mean	Range
Human	8	10.6	6.95 – 24.4
Dog	4	4.2	3.5 – 4.9
Rabbit	9	1.6	0.7 – 2.7
Guinea pig	6	1.5	0.6 – 2.6
Ferret	6	1.1	0.5 – 1.7
Burro	1	0.24	
Horse	3	0.13	0.08 – 0.18
Mouse	13	0.072	0.04 – 0.11
Hamster	3	0.022	0.017 – 0.031
Rat	3	0.0054	0.004 – 0.008
Goat	9	0.0035	0.39 –< 0.00002
Sheep	2	0.0022	0.0016– 0.0030
Chicken	4	0.00007	0.0002–< 0.00002

[a]SPA-binding capacity in human IgG equivalents/ml. One human IgG equivalent is defined as an SPA-binding capacity equal to that of 1 mg of the purified human IgG standard, as measured by inhibition of the binding of [^{125}I]SPA to rabbit IgG immobilized on polyacrylamide beads

1978). These IgE molecules apparently bind via their Fab portion, rather than their Fc portion (*Johansson* and *Inganas* 1978; *Inganas* et al. 1981). The biological significance, if any, of this unusual interaction of a small proportion of a minor class of immunoglobulin with SPA remains to be elucidated. Several other studies of the subclass binding specificities of the sera from various animal species have been published (*Forsgren* 1968; *Kronvall* et al. 1970c; *Medgyesi* et al. 1978; *Goudswaard* et al. 1978; *Duhumel* et al. 1980; *Coe* et al. 1981). Of most general interest, mouse IgG2A, IgG2B, and IgG3 bind well, whereas IgG1 binds less well, and the majority of IgA and IgM do not bind (*Kronvall* et al. 1970c; *Chalon* et al. 1979).

Since SPA binds to the Fc portion of the immunoglobulin molecule (*Forsgren* and *Sjoquist* 1966; *Kronvall* and *Frommel* 1970), an important practical consequence of this binding site is that the attachment of SPA to an immunoglobulin molecule does not interfere with antigen binding by the Fab portion. Conversely, antigen binding probably does not induce a conformational change in the Fc portion of the immunoglobulin molecule and thus does not appear to modify SPA binding; however, enhanced interaction between antigen, antibody, and SPA may occur if complexes are generated with polyvalent antigens (*Kessler* 1976; *Sandor* and *Langone* 1981). The equilibrium constant for the reaction between SPA and two human myeloma proteins was 4×10^{-7} Mol^{-1}, which is equivalent to that of a low-affinity antigen-antibody reaction (*Kronvall* et al. 1970b; *Johnsson* and *Kronvall* 1974; *Kessler* 1975). Of additional practical importance, β_2-microglobulin, which contains a portion structurally analagous to the Fc fragment, does not bind SPA (*Stewart* et al. 1978).

The SPA-binding domain on the Fc portion has been further localized to the C_H2–C_H3 interface, and binding of SPA requires the integrity of all or at least portions of both of these domains (*Lancet* et al. 1978; *Stewart* et al. 1978; *Deisenhofer* et al. 1978). The SPA-binding site is distinct from the C1-binding site of the C_H2 domain (*Lancet* et al. 1978; *Deisenhofer* 1981); however, steric hindrance may result in competitive blocking of C1 binding by SPA (*Stalenheim* et al. 1973; *Langone* 1978). Whether SPA binding to immunoglobulin inhibits or activates serum complement depends upon the ratios of the two proteins. At ratios of at least two immunoglobulin molecules to one SPA molecule, complexes are generated that behave functionally like IgM in their heightened ability to fix complement (*Sjoquist* and *Stalenheim* 1969; *Langone* et al. 1978).

5 Potential Advantages of SPA over Antiglobulin

Staphylococcal protein A offers several potential advantages over antiglobulin, one or more of which may prove useful in specific situations (Table 3). SPA is a well-defined molecule that is readily available in pure form, either commercially or as a result of relatively simple laboratory preparations. SPA is a highly stable molecule that can be conjugated to a number of marker molecules with little or no loss of activity (Table 1). SPA reacts with the immunoglobulins of most mammalian species (*Kronvall* et al. 1970a; *Kessler* 1976; *Goding* 1978; *Richman* et al. 1982). This permits indirect antibody assays with sera for which antiglobulins are not readily available (*Richman* et al. 1982). This property also permits the detection of multiple antigens in indirect immunoassays utilizing antisera from several different species (*Richman* et al. 1982, to

Table 3. Potential advantages of SPA over antiglobulin

1. SPA is readily available in pure form.
2. SPA is a highly stable molecule under a variety of conditions.
3. SPA reacts with the immunoglobulins of most mammalian species.
4. SPA can be conjugated with a number of marker molecules with little or no loss of immunoglobulin-binding activity.
5. SPA generally exhibits low nonspecific binding to materials and reagents.
6. SPA is not bound by Fc receptors present on herpesviruses and herpesvirus-infected cells.

be published a). For example, detection and identification of four different human herpesviruses has been carried out with an indirect enzyme immunofiltration assay that utilized rabbit antisera, human serum, guinea pig antiserum, and mouse monoclonal antibodies, all detected with the same SPA-horseradish peroxidase conjugate (Fig. 1).

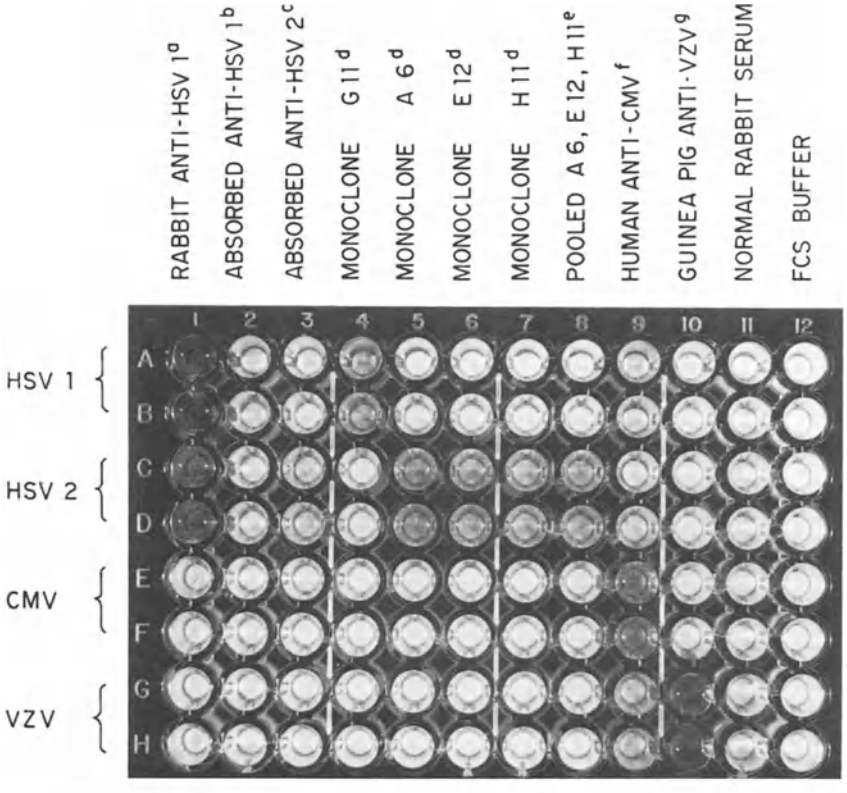
The tendency of SPA to bind nonspecifically to materials such as plastic, filters, cells, and uncomplexed antibody appears to be lower than that of antiglobulins (*Kessler* 1975; *Brown* et al. 1977; *Zeltzer* et al. 1978; *Engvall* 1978; *Cleveland* et al. 1979). Some of this lower nonspecific binding may be due to differences in the relative intrinsic, nonspecific reactivities of SPA and immunoglobulin molecules; some may be attributable to the fact that, theoretically, all labeled SPA molecules are functional whereas only a small fraction of the labeled immunoglobulin in an antiserum contains the specific antibody desired. This latter aspect may be obviated with the advent of monoclonal antibody reagents.

Finally, SPA does not bind to the Fc receptors induced in cells by several herpesviruses (*Yasuda* and *Milgrom* 1968; *Furukawa* et al. 1975; *Rahman* et al. 1976; *Sakuma* et al. 1977; *Costa* et al. 1978). These receptors, in the case of herpes simplex virus (HSV) types 1 and 2, were recently shown to be the virion glycoprotein E (*Para* et al. 1982a, b), which one could speculate might benefit the virus in the same way that SPA itself may benefit the staphylococcus.

Antiglobulin possesses several potential advantages over SPA. The former may react with certain classes and subclasses of immunoglobulins such as IgG3 or IgM, which may be important in a particular assay. Moreover, serum immunoglobulins of certain animals (sheep, goat, or bird, for example) may react very poorly with SPA. A specific antiglobulin of particularly high affinity may provide greater sensitivity than SPA. Whether SPA or antiglobulin will prove more useful in any particular situation will often require comparison in empirical trials.

6 Applications of SPA for Immunoassays

Staphylococcal protein A can be used advantageously in many ways in the development and performance of immunoassays (Table 4). Affinity chromatography with SPA-conjugated Sepharose represents one of the most simple and effective methods of purifying and concentrating immunoglobulins (*Hjelm* et al. 1972; *Goding* 1976). Purifying antibody prior to conjugation with labeling molecules increases the specific



HSV 1	A	1.17	.12	.06	.34	.00	.00	.00	.00	.22	.02	.05	.00
	B	1.17	.10	.04	.32	.00	.00	.00	.00	.20	.01	.05	.00
HSV 2	C	.77	.02	.18	.00	.40	.29	.25	.26	.16	.01	.04	.00
	D	.86	.02	.16	.00	.40	.30	.26	.26	.15	.02	.04	.00
CMV	E	.08	.00	.04	-.01	.01	.00	-.01	-.01	.53	.03	.04	.01
	F	.07	.00	.00	.00	-.01	-.01	-.01	.01	.56	.00	.03	.00
VSV	G	.07	.00	.00	-.01	-.01	-.01	-.01	-.01	.16	.99	.05	.00
	H	.08	.00	.01	-.01	-.01	.00	-.01	-.01	.15	.94	.05	.01

Fig. 1. Enzyme immunofiltration assay of various antisera and monoclonal antibodies to human herpesvirus antigens. A single antiserum or antibody dilution was used in each vertical column of wells. One of four different virus antigens was used in a pair of horizontal rows of wells. In the *photograph*, the reactions are apparent by the enzymatic conversion of substrate to a colored product. The *table* gives the absorbance reading at 488 nm in each well as measured in a micro-Elisa reader after zeroing the spectrophotometer on well *A12*, which contained fetal bovine serum buffer and HSV-1-infected cells. The dilutions of each serum or antibody in fetal calf serum buffer were: *a*, 1:500; *b*, 1:5000; *c*, 1:10 000; *d*, 1:8000; *e*, pool of three monoclonal antibodies each at 1:24 000; *f*, 1:5000; *g*, 1:3000. The absorbed antisera used in rows *B* and *C* are HSV-1 and HSV-2 convalescent rabbit sera that were absorbed against 3500 cells infected with the heterologous HSV serotype. *Richman* et al. (to be published a)

Table 4. Applications of SPA for immunoassays

1. Purification and concentration, by affinity chromatography, of immunoglobulins from antisera or from hybridoma cultures
2. Separation by affinity chromatography of Fab and $F(ab')_2$ fragments from Fc fragments or whole immunoglobulin molecules
3. Immunoadsorption for antigen detection or purification
4. Detection by indirect immunoassay of specific antigens or antibodies, using liquid-phase, solid-phase, or cytological assay techniques

activity of the conjugate and reduces nonspecific labeling. In our laboratory this technique reduces the protein content of antisera by 97%–99% and of ascites preparations of monoclonal antibodies by 92%–96% with recovery of 50%–90% of the specific antibody activity (unpublished observations). Affinity chromatography is also an extremely effective and easy method for purifying Fab fragments from Fc fragments and intact immunoglobulin molecules (*Hjelm* et al. 1972; *Goding* 1976).

Another application of SPA as an immunoadsorbent is antigen separation and purification using SPA in pure form or bound to Sepharose beads or to the intact staphylococcal cell (*Kessler* 1981; *Hayes* et al. 1978; *MacSween* and *Eastwood* 1978, 1981; *O'Keefe* and *Bennett* 1980; *Reisberg* and *Rossen* 1981).

Staphylococcal protein A has been used as a convenient and practical method for screening hybridomas for antibody production (*Nowinski* et al. 1979; *Buchanan* et al. 1981). The advantage of such a selection technique is that the monoclonal antibodies that result from such a process are easy to purify, manipulate, and detect in immunoassays that employ SPA.

Finally, SPA has found many applications in indirect immunoassays as a conjugate with the various molecules listed in Table 1. These assays include immunocytological techniques to detect antigens in cells by light microscopy (*Notani* et al. 1979; *Celio* et al. 1979), fluorescence microscopy (*Ghetie* et al. 1974a), transmission electron microscopy (*Bachi* et al. 1977; *Dubois-Dalcq* et al. 1977; *Hooghe-Peters* et al. 1979; *Geuze* et al. 1981), or scanning electron microscopy (*Hooghe-Peters* et al. 1979; *Pringle* and *Parry* 1980). These applications also include both solid-phase and immunoprecipitation assays for either antigen or antibody, as summarized below.

7 Use of SPA in Assays for Viral Antibodies

Using ^{125}I- or enzyme-labeled SPA, several immunoassay configurations have been designed to detect antibodies to hepatitis B virus (*Figenschau* and *Ulstrup* 1974) HSV (*Cleveland* et al. 1979), varizella-zoster virus (VZV) (*Richman* et al. 1981a), cytomegalovirus (CMV) (*Madore* and *Baumgarten* 1979), alphaviruses (*Jahrling* et al. 1978), rabies virus (*Atanasiu* and *Perrin* 1979), influenza virus (*Richman* et al. 1981b) Lassa virus (*Richman* et al. 1982), Ebola virus (*Richman* et al. 1983), Marburg virus (*Richman* et al., to be published), and murine retroviruses (*Colombatti* and *Hilgers* 1979; *Holmes* et al. 1980). The capacity of SPA to react with immunoglobulins from a wide variety of animal species has been exploited to establish antibody

assays against a number of animal viruses for which serological assays in multiple species are needed (*Soergel* et al. 1978; *Bommeli* et al. 1980; *Crowther* and *Abu Elzein* 1980; *Potgieter* et al. 1980).

Efforts to develop IgM-specific antibody assays have attempted to exploit the immunoglobulin class specificity of SPA. These IgM-specific assays are based upon the rationale that adsorption of a serum with SPA by one of several techniques eliminates the majority of IgG and thus makes it possible to attribute any residual antibody activity in the adsorbed serum to IgM. Many such assays have been developed for IgM antibody to rubella, a potentially extremely valuable assay for the diagnosis of congenital rubella or acute rubella infection using a single specimen of serum (*Kangro* et al. 1978; *Leinikki* et al. 1978; *Skaug* and *Gaarder* 1978; *Inouye* et al. 1978; *Crovari* et al. 1979; *Jankowski* et al. 1979; *Tuomanen* and *Powell* 1980; *Reiner* and *Wecker* 1981; *Braun* et al. 1981). A similar approach for IgM antibody to hepatitis A virus has been developed to permit the diagnosis of recent disease (*Bradley* et al. 1979). Although this approach is extremely attractive for its convenience and simplicity, it does not select for IgM antibody; it selects against SPA-reactive antibody. SPA adsorption fails to remove IgG3, IgA1, and IgE as well as IgM. Beck has shown that IgG3, although comprising only a small fraction of IgG, may contain a quantity of antibody to rubella, polio, or herpes simplex virus that is equal to or greater than its proportional representation in the serum (*Beck* 1981). Consequently, the use of protein A adsorption has been shown to yield false-positive antibody titers of alleged IgM specificity to rubella virus (*Crovari* et al. 1979; *Field* et al. 1980), hepatitis A virus (*Lofgren* et al. 1980), and toxoplasma (*Filice* et al. 1980).

8 Use of SPA in Assays for Viral Antigens

As with assays for antibody, ^{125}I- or enzyme-labeled SPA has been used in several immunoassay configurations to detect the antigens of hepatitis B virus (*Figenschau* and *Ulstrup* 1974; *Inman* et al. 1981), SV40 (*Crawford* and *Lane* 1977), the human herpesviruses (*Cleveland* et al. 1979; *Bystricka* et al. 1980; *Yolken* and *Stopa* 1980; *Cleveland* et al. 1981; *Richman* et al., to be published a), mouse mammary tumor virus (*Callis* and *Ritzi* 1981a, b), rotavirus (*Yolken* and *Leister* 1981), influenza A and B viruses (*Richman* et al., to be published a), measles virus (*Hayes* et al. 1978), respiratory syncytial virus (*Pringle* and *Parry* 1980), vesicular stomatitis virus (*Huang* and *Okorie* 1978), Sindbis virus (*Enzmann* 1978), and Ebola and Marburg viruses (*Richman* et al., to be published b). The Ebola virus immunoassay was used to demonstrate that virus isolates from two discrete outbreaks of Ebola hemorrhagic fever that occurred in 1976 were antigenically distinct (*Richman* et al., to be published b). *Inman* et al. (1981) reported methods utilizing SPA to isolate and characterize circulating immune complexes from patients with hepatitis B systemic vasculitis. Together with specific antisera, SPA has also been used to fix and to agglutinate virions for electron-microscopic visualization (*Gough* and *Shukla* 1980; *Hebert* et al. 1981).

A recent application of SPA has been the detection of electrophoretically separated virion proteins that have been transferred to paper, i.e., "Western blotting." Proteins from virion or infected cell preparations are separated by standard sodium dodecyl sulfate – polyacrylamide gel electrophoresis. The proteins are then electro-

phoretically transferred from the gel to nitrocellulose or diazobenzyloxymethyl paper. Proteins are detected by incubation with antiserum and then with radioiodinated SPA. In addition to exploiting the powerful separation technique of gel electrophoresis, this approach provides the advantages of the immunological identification of specific proteins in an extremely impure mixture, and the ability to detect impure and minority proteins without having to metabolically radiolabel them. Radioiodinated SPA has been used for "Western blots" in order to analyze the proteins of HSV (*Norrild* et al. 1981), murine retroviruses (*Burnette* 1981), and SV40 (*Reiser* and *Wardale* 1981). SPA also provides the advantage of allowing simultaneous use of antibodies from multiple species, e.g., rabbit polyvalent antiserum and mouse monoclonal antibodies. In our laboratory we have recently been developing such blots with biotinylated-SPA followed by avidin-biotin-horseradish peroxidase complexes. This technique using SPA-enzyme has proved to be as sensitive as the technique using radioiodinated SPA, but it eliminates the need for radiochemicals and photographic development. The results are immediate and visually interpretable (unpublished observations).

9 Use of SPA in Rapid Viral Diagnosis

Several of the viral immunoassays described in the preceding two sections can be performed in a matter of hours and have been applied to clinical situations. Several assays that were developed in our laboratory will be described to illustrate applications of SPA to rapid viral diagnosis. The first application involves the rapid detection of antibody to VZV. Because of the morbidity and mortality of varicella in immunosuppressed patients and the risk of epidemic nosocomial infection, it is extremely important to be able to determine rapidly whether immunosuppressed children and hospital personnel who are exposed to varicella are immune or susceptible to infection. We adapted our indirect [^{125}I]SPA immunofiltration assay (*Cleveland* et al. 1979) to detect antibody to VZV, resulting in a highly sensitive and specific test for immunity to varicella (*Richman* et al. 1981a). Using this assay, a single technician can easily determine the presence or absence of antibody to VZV in more than 30 sera in half a day. As little as 30 μl of serum is required per patient. The necessary VZV and control antigens are prepared in large batches and stored in aliquots, which permits the assay to be run on demand (*Zaia* and *Oxman* 1977). This assay, which has proved to be of much practical use in outbreaks of varicella in hospital wards with cancer patients, has subsequently been converted to an SPA conjugated horseradish peroxidase assay and has been adopted for use by several other laboratories.

This indirect enzyme immunoassay has also been used to identify and characterize the virus that is producing cytopathic effects (CPE) in tube cell cultures inoculated with clinical specimens. A single tube culture showing viral CPE is frozen on a slant and the contents are then swirled with a vortex mixer while thawing. The resulting suspension is then used as antigen in the immunofiltration assay, with sequential incubations and washes with antisera, SPA-horseradish peroxidase, and colorigenic substrate. A visually interpretable result is obtained, permitting the identification of an isolate within 3 h (Fig. 1). This assay, which can utilize antisera from various species as well as monoclonal antibodies, is being used routinely in our laboratory to identify isolates of HSV, VZV, CMV, and antigenic variants of influenza A and

B virus (*Richman* et al. 1981b, to be published a). In addition, more than 500 clinical isolates of HSV have been unequivocally serotyped with this assay, using type-specific monoclonal antibodies (*Richman* et al., to be published).

More recently we have applied this technique to the direct detection of HSV antigens in eye swabs from rabbits with primary herpes keratoconjunctivitis (*Cleveland* et al., to be published). Using calcium alginate swabs, HSV antigens were detected with 95% sensitivity and complete specificity, using virus isolation in cell culture as the standard of diagnosis. The results of this assay are visually interpretable in 2 h. The results of additional studies to detect viral antigens directly the rabbit model and in clinical specimens have been encouraging.

10 Conclusions

Staphylococcal protein A is a 42 000 molecular weight protein component of the cell wall of *S. aureus*. The capacity of SPA to bind to the Fc portion of many classes of immunoglobulin of most mammalian species has thrust SPA into prominence as an extremely useful reagent for both immunochemistry and immunoassays.

The location and role of SPA in the staphylococcus, the biochemical characteristics of SPA, and the reactivity of SPA with immunoglobulins with regard to animal species, immunoglobulin subclass, and site on the immunoglobulin molecule have been reviewed.

SPA may offer several potential advantages over antiglobulin, one or more of which may prove useful in specific situations. These include availability in pure form, high stability, reactivity with the immunoglobulins of a wide range of mammalian species, ability to be conjugated with a wide array of marker molecules with little or no loss of immunoglobulin binding activity, low nonspecific binding to materials and reagents, and nonreactivity with Fc receptors.

Staphylococcal protein A has thus proven extremely useful for the purification and concentration of immunoglobulins, for the purification of Fab fragments from Fc fragments and immunoglobulins, for antigen separation and purification by immunoadsorption, for screening hybridomas for antibody production, and in the development of many indirect immunoassays. These assays for either antigen or antibody, which include solid-phase, liquid-phase, and immunocytological techniques, have been summarized. Finally several applications of SPA for rapid viral diagnosis have been described.

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Use of Monoclonal Antibodies for Viral Diagnosis

ROBERT H. YOLKEN*

1 Introduction

The performance characteristics of immunoassay systems are dependent to a great extent on the binding characteristics of the immunoreactants utilized. For this reason, investigators involved in the use of immunoassay systems for the detection of microbial antigens have been particularly interested in the evolving technology related to the production and development of monoclonal antibodies (*Kennet* et al. 1980;

Table 1. Application of monoclonal antibodies in viral diagnosis: advantages and problems

Advantages
Consistent supply of reagents
Defined sensitivity and specificity
Distinct reactions with viral and host components
Practical measurement of viral characterization
Increased proportion of immunoglobulin directed against antigen
Problems
Narrow range of reactivity
Unfavorable affinity characteristics
Undefined reaction conditions
Variable expression of nonimmune functions
Biological cross-reactivities

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Köhler and *Milstein* 1975; *Steinitz* et al. 1977). The potential advantages of monoclonal antibodies for immunoassay systems are numerous (Table 1). For example, a consistent supply of a well-defined reagent should allow for the wide-scale availability of immunoassay systems with defined performance characteristics. In addition, since immunoassays follow the law of mass action (Table 2), the availability of immunoglobulin preparations with every molecule directed against antigen should improve the kinetics and thus the sensitivity of immunoassay systems (*Yolken* 1982). This is especially true in the case of solid-phase immunoassays since the concentration of immunoglobulin which can be utilized in these systems is limited by the binding properties of the solid phase. The specificity of the monoclonal antibodies also allows the utilization of competitive and homogeneous immunoassays which are difficult, if not impossible, to perform with polyclonal antibodies due to the high amount of nonreactive immunoglobulin in such preparations. In addition, the specificity of monoclonal antibodies allows the performance of specific serotyping reactions and measurements of antigenic variations using fairly simple procedures. Finally, the ability to produce antibodies directed specifically against viral antigens but not against host components should allow the development of immunoassay systems with few, if any, false-positive reactions due to the presence of nonviral components in the clinical

Table 2. Properties of antigen-antibody interactions applicable in enzyme immunoassay

I. Law of mass action

$$\frac{(AgAb)}{(Ag)\,(Ab)} = K_a$$

II. Theoretical equilibrium values

K_a	(Ab)[a]	Fraction of antigen bound
10^8	10^{-7}	0.90
(High-affinity antibody)	10^{-8}	0.50
	10^{-9}	0.09
	10^{-10}	0.01
10^6	10^{-7}	0.09
(Low-affinity antibody)	10^{-8}	0.01
	10^{-9}	0.001
	10^{-10}	0.0001

III. Attainable solid-phase antibody concentrations

Antibody type (%)	(Ab)[a]
Monoclonal (100)	10^{-7}
Affinity purified (30)	3×10^{-8}
Hyperimmune (10)	10^{-8}
Postinfection (1)	10^{-9}

Abbreviations: $(AgAb)$, concentration of antigen-antibody complex (moles/liter); (Ag), concentration of unbound antigen; (Ab) concentration of unbound antibody; K_a, affinity constant of antigen-antibody reaction

[a]Assuming a maximal binding capacity of 10 µl/ml and a molecular weight of 100 000

specimen. This situation is in contrast with immunoassay systems which utilize serum from immunized animals, since such sera often contain antibodies to nonviral antigens (*Yolken* 1980). These nonspecific reactivities are due to naturally occurring antibodies as well as to contaminating antigens in the immunizing preparation.

While there are a number of obvious advantages related to the use of monoclonal antibodies, there are some theoretical disadvantages which must be addressed before monoclonal antibodies can be advocated as general reagents in immunoassay systems (*David* et al. 1981; *Gerhard* et al. 1978; *Laver* et al. 1979) (Table 1). The most important is that many monoclonal antibodies might be directed against a range of antigens which is so narrow that they might not react with antigens present in clinical specimens from infected individuals. The use of such antibodies might thus lead to false-negative reactions in immunoassay systems. Another possible problem with monoclonal antibodies is that since they only react with a single antigenic site, they might be difficult to use in "sandwich" immunoassays, which are based on the binding of antigen to solid-phase and liquid-phase antibody. In addition, it is possible that individual monoclonal antibodies might fail to react with plastic supports, covalent linkers, or complement components in a manner similar to that of polyclonal antibodies. Finally, there has been some suggestion that the affinity constants of monoclonal antibodies might be less than those of polyclonal antibodies directed against the same antigen (*Frankel* and *Gerhard* 1979). Since the sensitivity of solid-phase immunoassays is highly dependent upon affinity, the decreased affinity might tend to counterbalance some of the advantages inherent in the higher antibody concentration offered by monoclonal antibodies (Table 2).

2 Production of Monoclonal Antibodies

The strategies for the production of monoclonal antibodies have been reviewed in detail in recent publications (*Galfré* and *Milstein* 1982; *St. Groth* 1980; *St. Groth* and *Scheidegger* 1980).

One area which we have found to be important in the generation of efficient reagents for immunoassay systems involves the screening of clone supernatants for antibody activity. We found it to be particularly important that the screening method utilized be one which selects for high-affinity antibody. In the case of viral antigens, solid-phase radioimmunoassays or enzyme immunoassays are particularly useful since the assays are convenient and tend to select for high-affinity antibodies. On the other hand, immunofluorescence and neutralization assays will also detect antibodies of lower affinity. Although these antibodies are useful for certain purposes, the low-affinity antibodies are generally not suitable for use in the development of sensitive solid-phase immunoassay systems. Another point which we have found to be important concerns the production of large amounts of antibodies. We have found that, in some cases, the generation of large amounts of antibodies by inoculation of the monoclone into mouse ascites fluid leads to the generation of antibody with decreased reaction performance. This is undoubtedly due to the fact that the monoclonal antibody becomes diluted by mouse-derived antibodies in the ascites fluid. In addition, the use of ascites fluid can lead to nonspecific reactions due to heterotypic, mouse-derived antibodies. Although these problems can be overcome by the use of nude

mice to generate the ascites fluid, such nonantibody-producing mice are not widely available. An alternative is to produce larger amounts of tissue culture supernatants and to concentrate the antibody by the use of pressure dialysis or similar methods. The availability of liposome methods for production of large amounts of monoclonal antibody from tissue cultures should make this approach more practical.

3 Immunoassays Utilizing Monoclonal Antibodies

Although monoclonal antibodies can be utilized in a wide variety of immunoassays including radioimmunoassays and immunofluorescent assays, our laboratory has concentrated on adapting monoclonal antibodies to enzyme immunoassay technology. We prefer to use enzyme immunoassays due to the fact that these assays use stable reagents and can thus take maximal advantage of the high consistency offered by the monoclonal antibody technology (*Voller* et al. 1976; *Wisdom* 1976). In addition, enzyme immunoassays offer a high sensitivity due to the inherent magnification of enzyme-substrate reactions. This sensitivity is particularly evident when high-energy substrates such as those generating fluorescent, chemiluminescent, or radioactive products are used.

4 Labeling of Monoclonal Antibodies

Enzyme immunoassays involve the use of enzymes as immunoglobulin markers. We have found that most monoclonal antibodies can be covalently linked with enzymes such as alkaline phosphatase, β-galactosidase, or horseradish peroxidase, applying procedures identical to those used with polyclonal antibodies (Table 3). For example, glutaraldehyde can be used to link monoclonal antibodies to enzymes containing ε-amino groups such as alkaline phosphatase (*Engvall* and *Perlmann* 1972) or peroxidase (*Avrameas* and *Ternynck* 1971), sodium metaperiodate can be used to couple antibodies to glycoprotein enzymes such as horseradish peroxidase (*Nakane* and *Kawaoi* 1974), and thiol-reacting agents can be used to link monoclonal antibodies to SH-containing enzymes such as bacterial β-galactosidase (*Sullivan* and *Marles* 1982). However, we have found that, as is the case with polyclonal antibodies, the enzyme labeling process can be difficult to control and can lead to batch-to-batch variation in the activity of the conjugates. This variability negates some of the consistency inherent in the use of monoclonal antibodies. In addition, the enzyme-antibody conjugated often exhibit less antigen-binding capacity than the unlabeled antibodies due to the high molecular weight of the conjugate (*Boursma* and *Kalsbeek* 1975).

One approach to these problems is to use low molecular weight markers to label the monoclonal antibodies. After the reaction of the labeled antibody with antigen, enzyme so modified as to react with the low molecular weight marker is added. Two markers which we have found to be particularly useful are biotin and fluorescein. These markers were chosen since reproducible methods for covalently linking these markers with free amino groups of immunoglobulin are available (*Guesdon* et al. 1979; *Hofman* et al. 1977; *Nakamura* 1980). For example, biotin can be simply linked to antibody by reacting the antibody with biotin *N*-hydroxysuccinimide ester. In the case

Table 3. Comparison of enzymes available for enzyme immunoassay

Enzyme	Source	Uses	pH optimum	Specific activity (units/mg)	Molecular weight	Practical methods of conjugation[a]	Practical substrates available		
							Visual	Fluorescent	Radioactive
Alkaline phosphatase	Calf intestine	EIA	8–10	400	100 000	Glutaraldehyde, 1 step	NP-PO_4	MU-PO_4 FM-PO_4	[^{3}H]AMP
Peroxidase	Horseradish	EIA, HIST	5– 7	900	40 000	Glutaraldehyde, 1 step	H_2O_2 + 5-AS H_2O_2 + OPD	NADH HPA	NA
						Glutaraldehyde, 2 step, $NaIO_4$	H_2O_2 + ABTS		
β-galactosidase	*Escherichia coli*	EIA	6– 8	400	540 000	Glutaraldehyde, 1 step *p*-Benzoquinone *N-N'*-OPLD	NP-Gal	MU-Gal	[^{3}H]GAlP
Glucose oxidase	*Aspergillus niger*	EIA, HIST	4– 7	200	160 000	Glutaraldehyde, 1 step	Glu + 5-AS Glu + NBT Glu + MTT	NADH HPA	[^{3}H]Glu
Catalase	Calf liver	EIA	6– 8	40 000	250 000	Glutaraldehyde, 1 step (SPA)	H_2O_2[b]	NA	NA

Abbreviations: EIA, enzyme immunoassay; NP-PO_4, *p*-nitrophenyl phosphate; MU-PO_4, methyl umbelliferyl phosphate; FM-PO_4, fluorescein methyl phosphate; HIST, enzyme-mediated histochemical procedure; 5-AS, 5-aminosalicylic acid; NADH, nicotinamide adenine dinucleotide phosphate, reduced form; NA, not available; OPD, *o*-phenylene diamine; HPA, *p*-hydroxyphenylacetic acid; ABTS, 2,2-azino-di-(3-ethylbenzothiazoline sulfone-6) diammonium salt; NP-Gal, nitrophenyl galactose; MU-Gal, methyl umbelliferyl galactose; [^{3}H]GalP, [^{3}H]galactose-6-phosphate; *N,N'*-OPLD, *N,N'-o*-phenylene dimaleimide; Glu, glucose; [^{3}H]Glu, [^{3}H]glucose; NBT, *p*-nitroblue tetrozolium chloride; MTT, thiazolyl blue; SPA, staphylococcal protein A

[a]See Avreameas et al. (1978) for description of methods; [b]Measured spectrophotometrically at 240 nm

of biotin-labeled antibodies, the reaction is quantitated by the addition of avidin, either linked covalently with enzyme or bound to biotinylated enzyme in the form of an avidin-biotin complex (*Warnke* and *Levy* 1980). In the case of fluorescein, the reaction is quantitated by the addition of enzyme-labeled antibody directed against this hapten. The use of the avidin-biotin system has the advantages of not introducing an additional immunoglobulin species into the system and of providing some additional magnification at the level of the avidin-biotin interaction. This can result in assay systems which are more sensitive than the ones which utilize enzyme-labeled antibody directly. We have found that the process of labeling monoclonal antibodies with small molecules such as biotin is extremely reproducible and that conjugates of uniform performance characteristics can be consistently prepared. An alternative approach to the problem of labeling involves the use of indirect systems which do not utilize labeled antiviral antibody but which make use of labeled antibodies directed against the species of immunoglobulin. These systems are discussed in detail below.

5 Assay Formats

Monoclonal antibodies can be utilized for solid-phase immunoassays in a number of different formats (*Kapikian* et al. 1979; *Yolken* 1980; *Yolken* et al. 1980). One of these formats is that of direct immunoassay depicted in Fig. 1. In this form of immunoassay, antibodies are bound to a solid-phase surface such as a plastic bead or a micro-

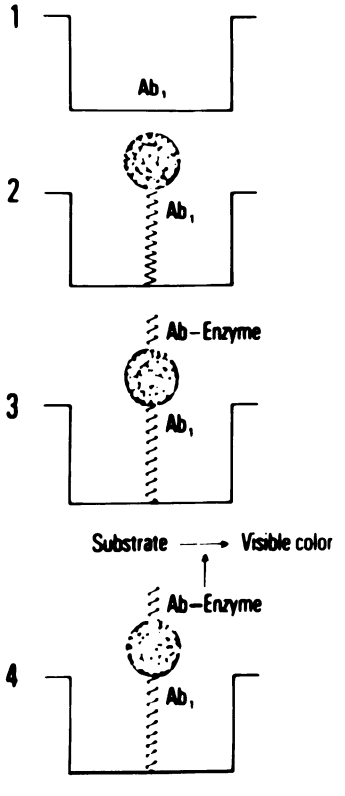

Fig. 1. Direct ELISA for antigen measurement. *1*, Antibody (Ab_1) directed against the antigen to to be measured is adhered to the well of a microtiter plate. *2*, The test material is added. Any antigen against which the antibody is directed will adhere. *3*, Antibody labeled with an enzyme is added. This will react with the antigen that is adhered to Ab_1. *4*, substrate is added. The enzyme adhered to the well will convert the substrate to a visible form. The amount of color measured is proportional to the amount of antigen in the test material

titer well. Specimen is then added to the antibody-coated solid phase. After the antigen-antibody reaction, unreacted antigen is removed by washing and enzyme-labeled antibody is added. Since this form of assay system requires binding of antigen at two sites, it is preferable that monoclonal antibodies with different reactivities be used to coat the solid phase and to prepare the liquid-phase antibody. However, we have found that for many viral antigens, the same monoclonal antibody can be used both as the solid- and liquid-phase antibody. This is probably a reflection of the fact that many viral antigens are present in polymeric form in body fluids, thus allowing binding to the solid phase and to labeled antibody through the same antigenic determinants.

Monoclonal antibodies can also be utilized to perform immunoassays in the indirect format (Fig. 2). In this format unlabeled antibody is added to the liquid phase, and the amount of bound antibody is quantitated by the addition of a labeled species (usually an antiglobulin) capable of reacting with liquid-phase antibody specifically

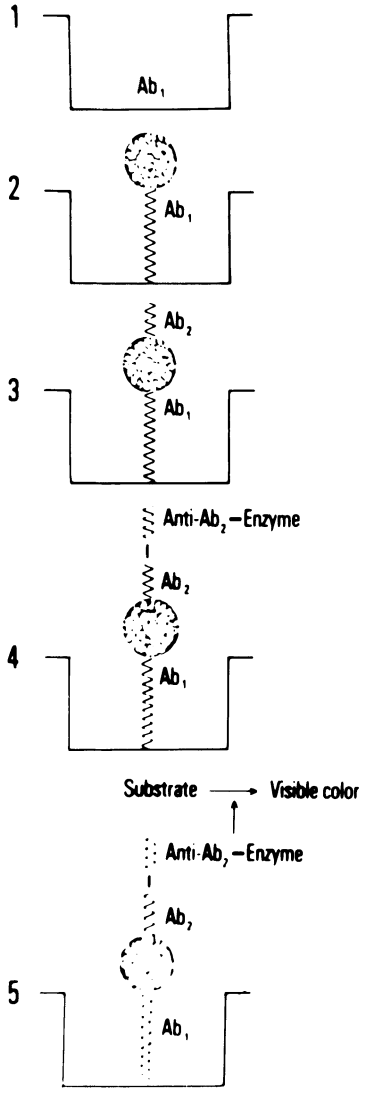

Fig. 2. Indirect ELISA for antigen measurement. *1*, Antibody (Ab_1) directed against the antigen to be measured is adhered to the well of a microtiter plate. *2*, The test material is added. Any antigen against which the antibody is directed will adhere. *3*, Unlabeled antibody (Ab_2) from a different animal than Ab_1 is added. This will react with any antigen that is adhered to Ab_1. *4*, Enzyme-labeled antibody directed against the globulins of the animal source of Ab_2 is added. *5*, A substrate is added. The enzyme adhered to the well will convert the substrate to a visible form. The amount of color measured is proportional to the amount of antigen in the test material

bound to solid-phase antigen. When this assay is used with polyclonal antisera, it is necessary that the antibody used to coat the solid phase and that applied in the liquid phase be prepared in different animal species to prevent nonspecific interactions of the antiglobulin with the solid-phase antibody. However, the uniform nature of monoclonal antibodies allows their utilization in this system in a number of additional ways. We have found, for example, that monoclonal IgM antibody can be used to coat the solid phase and that monoclonal IgG antibody can be used as the liquid-phase antibody. In this case, a labeled antiglobulin directed specifically against mouse IgG will only react with antibody bound in the liquid phase and hence with antigen. We have found that goat anti-mouse IgG purified by affinity chromatography can be employed for this purpose. In a similar manner, an antibody which does not bind protein A can be used to coat the solid phase, and a protein A-binding antibody can be used in the liquid phase. In this system, enzyme-labeled protein A can be employed to quantitate the antigen-antibody reaction. An alternate method of applying monoclonal antibodies in indirect assays is to use them in conjunction with polyclonal animal-derived antisera. This system is advantageous in cases where two high-affinity monoclonal antibodies are not available for use in the immunoassay system. We have found it particularly advantageous to use the monoclonal antibody as the solid-phase immunoglobulin, thus utilizing its high specific activity to counterbalance the limited binding capacity of the solid phase (Fig. 2, Table 1).

The specificity of monoclonal antibodies allows the performance of assay formats which are difficult, if not impossible, to perform with polyclonal antibodies. One format which is particularly suited to rapid diagnosis is the double-determinant assay depicted in Fig. 3 (*Brown* et al. 1981; *Yolken* and *Leister* to be published). In this form of assay, antigen is simultaneously bound to solid-phase antibody and labeled liquid-phase antibody. By selecting monoclonal antibodies directed against different sites, competition between the solid-phase antibody and the labeled antibody can be avoided. Thus, these reactions can be performed simultaneously. After completion of the antigen-antibody reactions, unreacted antibody is removed by washing and the amount of labeled antibody bound to the solid phase is quantitated. The principal advantage of this assay format is that it is very rapid, requiring only a single antigen-antibody incubation period and a single washing step. This test is very convenient and is particularly suited for use with a variety of solid-phase supports. The sensitivity of double-determinant assays can be equivalent to that of immunoassays requiring much longer incubation periods (*Yolken* and *Leister* to be published). These assays might thus represent an important improvement in practical immunoassay technology.

Enzyme immunoassays can also be performed in a homogeneous format, that is, one not requiring a reaction procedure to separate antigen-antibody complexes from unreacted antibody (*Rubenstein* et al. 1972). Homogeneous assays are based on the fact that antigen-antibody complexes can interfere with the enzyme-substrate reaction by steric hinderance. Although such homogeneous assays have been devised for the measurement of a number of small molecules such as drugs and steroid hormones, previously developed technology has not found wide use for assaying macromolecular antigens, since complexes with such antigens do not significantly interfere with the diffusion of low molecular weight substrates (*Rubenstein* 1978). However, we have been able to formulate homogeneous assays for macromolecular viral antigens using polymeric substrates. In these assays, the binding of antigen to enzyme-labeled anti-

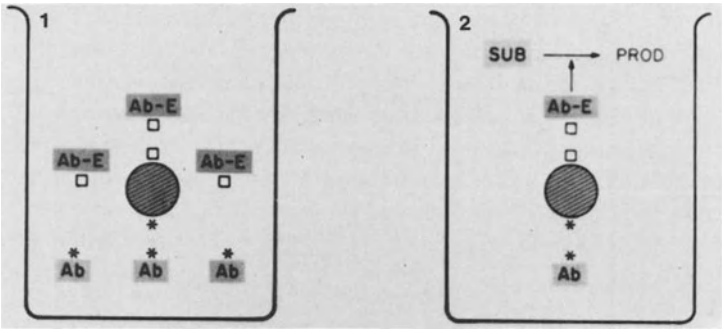

Fig. 3. Double-determinant enzyme immunoassay. *1*, Antigen is added to a solid phase coated with antibody directed against one antigenic site (* *Ab*). Enzyme-labeled antibody directed against a different site on the antigen (□ *Ab-E*) is then added. This will react with unbound sites on the antigen. *2*, Following a washing step to remove unreacted □ *Ab-E*, substrate is added. This will be converted by bound □ *Ab E* to a measurable product. The amount of product formed will be proportional to the concentration of antigen in the specimen

body will interfere with the interaction of the enzyme with the high molecular weight substrate (Fig. 4). One problem with these types of assays is that they are competitive in nature, that is, unreacted antibody leads to the generation of positive signals (*Gibbons* et al. 1980). Since a large percentage of the immunoglobulin in polyclonal antisera is not directed against antigen, competitive assays using such antibodies show a large amount of background activity. However, since virtually all of the labeled monoclonal antibodies are directed against antigen, the use of monoclonal antibodies in

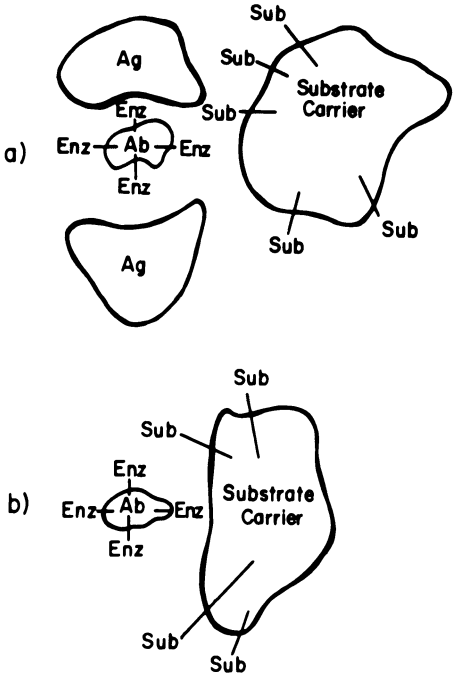

Fig. 4a, b. Homogeneous enzyme immunoassay for viral and bacterial antigens. a) If antigen (*Ag*) is present is a specimen, it will react with the antibody (*Ab*)-enzyme conjugate. When the macromolecular substrate is added, it will be prevented from reacting with the antigen-antibody-enzyme complex by steric hindrance. b) Uncomplexed antibody will react with available substrate to produce a measurable product. The amount of product is inversely proportional to the amount of antigen in a specimen

these competitive systems lead to a more favorable signal-to-noise ratio and to a more efficient assay system. Monoclonal antibodies are thus particularly suited to homogeneous assay systems. The availability of such systems might markedly simplify procedures for the detection of viral and other macromolecular antigens in body fluids.

Solid-phase immunoassays can also be performed in a competitive manner (*Belanger* 1978; *Segal* et al. 1979). One widely used system involves the interaction of labeled antibody with antigen in clinical specimens. The amount of unreacted antibody is then determined by reaction with a solid-phase antigen (Fig. 5). When polyclonal antibodies

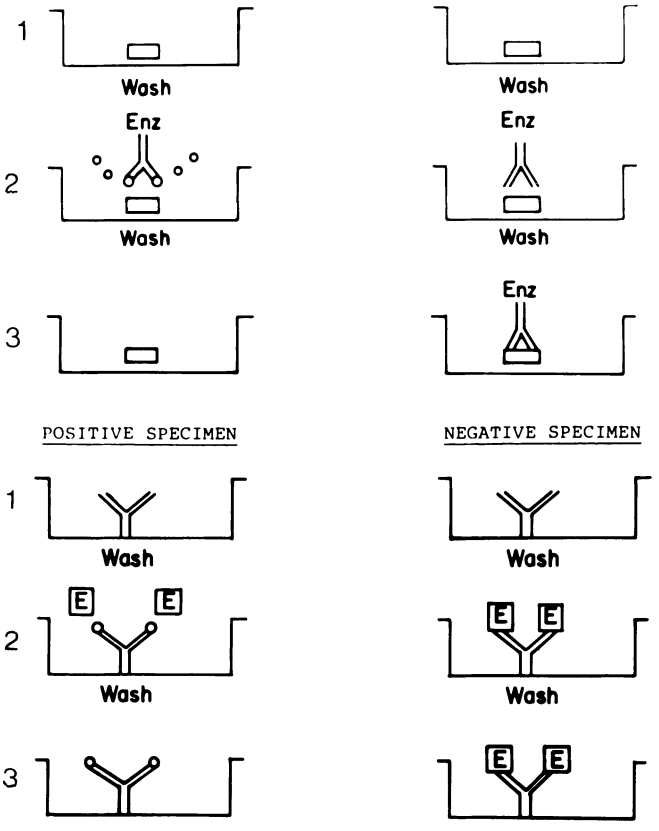

Fig. 5a. Competitive EIA using antigen bound to the solid phase. *1*, Antigen (□) is bound to the solid phase. Unbound antigen is removed by washing. *2*, The specimen is reacted with enzyme-labeled antibody ($\overset{ENZ}{\curlyvee}$) and the mixture is added to the solid phase. If the specimen contains antigen (○) it will react with the enzyme-labeled antibody, thus preventing the enzyme-labeled antibody from reacting with the solid phase. 3, Unbound enzyme-labeled antibody is removed by washing and the bound enzyme is quantitated by the addition of substrate. The amount of substrate product is inversely proportional to the amount of antigen in the specimen.
Unlabeled antibody can also be used in place of the enzyme-labeled antibody. In this case, enzyme-labeled antiglobulin or staphylococcal protein A is used to quantitate the antibody bound to the solid phase. b. Competitive EIA for antigen measurement – labeled antigen method. *1*, Antibody ($\curlyvee$) is bound to the solid phase. Unbound antibody is removed in the washing step. *2*, The specimen is added. If it contains antigen (○) it will bind to the solid-phase antibody. Enzyme-labeled antigen ([E]) is then added. This will react with antibody sites not occupied by antigen from the specimen. *3*, Unbound enzyme-labeled antigen is removed by washing, and the amount of bound enzyme-labeled antigen is quantitated by addition of the appropriate substrate. The amount of substrate product is inversely proportional to the amount of antigen in the test specimen

are used, the solid phase is washed and the amount of labeled antibody reacting with solid-phase antigen is determined. Thus, the presence of antigen in the initial specimen will be manifested by a decrease in antibody binding to the solid phase, and thus a decrease in the measurable signal. However, in the case of monoclonal antibodies, the fact that each labeled antibody is directed against antigen also allows the accurate determination of unreacted antibody by measuring activity in the liquid phase. By performing the assay in this way, a positive signal is generated, that is, antigen in the specimen will lead to an increased concentration of labeled antibody in the liquid phase and hence an increased amount of measurable color of fluorescence. The use of positive-signal measurements is more applicable to kinetic determinations and to visual reading than the reduced signal commonly employed in competitive assays. In addition, positive-signal assays have the potential for greater sensitivity since the signal is not greatly influenced by small variations in solid-phase antigen or labeled antibody concentrations.

One limitation of these competitive assays is the requirement for a large supply of antigen to be linked to the solid phase. The heterogeneous nature of many viral antigens makes it difficult to prepare aliquots of antigen which are absolutely consistent. The requirement for solid-phase antigen thus introduces variation into the systems. However, anti-idiotype competitive assay systems which totally avoid this problems have recently been described (*Potocnjak* et al. 1982). In this form of assay, monoclonal antibody is generated which is directed specifically against the idiotype of the antigen-specific monoclonal antibody. Since the anti-idiotype antibodies are directed against the part of the antibody which is involved in the antigen-antibody reaction, the formation of antigen-antibody complexes between the antiviral antibody and the antigen results in the exclusion of anti-idiotype antibody from the complex. The antigen-antibody reaction can thus be quantitated by measuring the amount of anti-idiotype antibody which does not bind to the complex. The advantage of this system is that it can measure specific antigen-antibody reactions without the need for any purified antigen. The production of anti-idiotype antibodies is performed by immunizing mice with the monoclonal antibody directed against the viral antigen and then screening clones for the ability to inhibit the antigen-antibody reaction between the antiviral antibody and the viral antigen. The sensitivity of this assay system is extremely high (10^{-12} mol/liter), probably due to the specificity and reproducibility of both the antigen-antibody and anti-idiotype-antibody reactions. In addition, this is a general assay system which could be applicable to the measurement of any antigen-antibody reaction.

Preliminary studies in our laboratory indicate that similar assays can be formulated employing polyclonal anti-mouse light chain antibody instead of the monoclonal anti-idiotype antibody. In these assay systems, the antigen-antibody complexes sterically inhibit the interaction of labeled anti-light chain antibody with the light chains of the solid-phase antiviral antibody. Since anti-light chain antibodies are more widely available than anti-idiotype antibodies, these assay systems might prove to be versatile for the measurement of antigen in clinical specimens. Studies in our laboratory have indicated that this format can be used to generate immunoassays which are more sensitive than those which involve the direct measurement of antigen-antibody reaction (*Yolken* 1982). The performance of these assays can be markedly improved by the use of monoclonal antibodies. For example, the coating of the solid phase with

a monoclonal antibody which does not fix complement can substantially reduce the amount of nonspecific reactivity in the system. In addition, the specificity of monoclonal antibodies is particularly important in these and other "magnified" immunoassay systems, since small amounts of nonspecific reactivity will result in the generation of a measurable signal. Finally, the availability of monoclonal antibodies directed at C3b and other complement components can markedly improve the specificity of the assay system by eliminating cross-reactions between complement components and noncomplement proteins such as immunoglobulin (Fig. 6).

6 Sensitivity

One important question concerning monoclonal antibodies is the sensitivity of immunoassay systems which use such antibodies as immunoreactants. We and others have found that many monoclonal antibodies can be employed to formulate sensitive

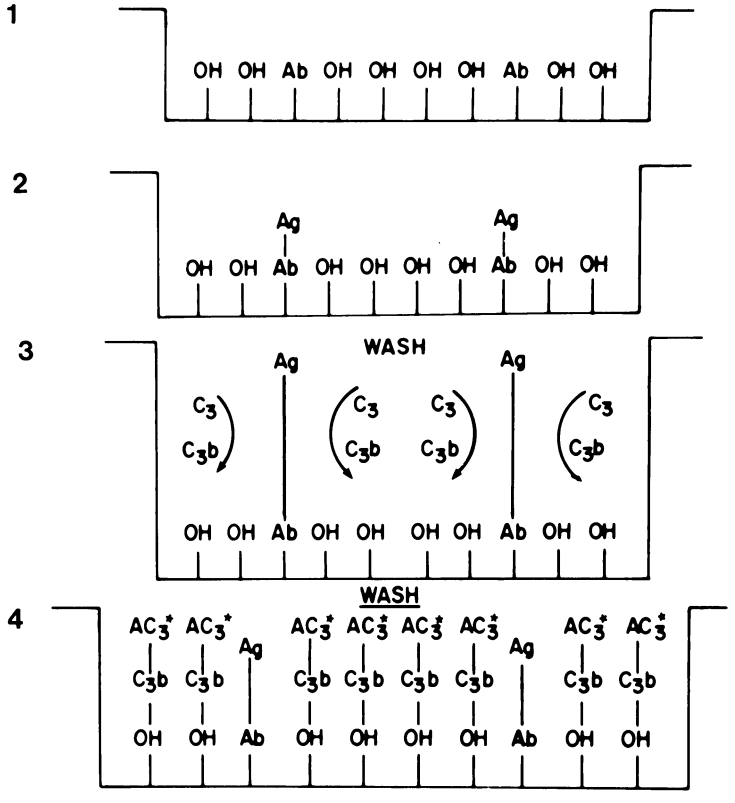

Fig. 6. Complement-magnified enzyme immunoassay. *1*, The solid phase is coated with antibody (*Ab*) directed against the antigen to be tested. The solid phase is also coated with a polysaccharide (*OH*) capable of binding activated complement. *2*, The specimen is added to the solid phase. Antigen (*Ag*) will bind to the antibody, and other material in the specimen will be removed by washing. *3*, A source of complement is added. The antigen-antibody complexes formed in *step 2* react with C_3 to produce an active fragment (C_3b). This fragment will bind to the polysaccharide bound to the solid phase. *4*, After unbound complement components are removed by washing, IgG directed against C_3 (AC_3) is added and quantitated

immunoassay systems. One particularly useful format involves the use of both monoclonal and polyclonal antibodies in the same system. In such systems, the most sensitive assays are often obtained by coating the solid phase with the monoclonal antibody and using the polyclonal antibody as the second reagent. The amount of antibody bound to the solid-phase antigen is quantitated by the addition of antiglobulin as shown in Fig. 2. This format takes advantage of the increased concentration of specific antibody on the solid phase offered by monoclonal antibodies, and the increased range of antigen-binding sites offered by the polyclonal antibodies. Specificity is insured by the lack of nonspecific binding of nonviral antigen to the solid-phase monoclonal antibody.

We have found that the sensitivity of immunoassay systems using monoclonal antibodies was greatly dependent upon the affinity of the antibody and the nature of the antigen. In the case of rotavirus (reagents supplied by Dr. H. Greenberg, National Institutes of Health, Bethesda, Maryland), cytomegalovirus (reagents supplied by Dr. Lenora Peirera, California State Health Department, Berkley, California), and

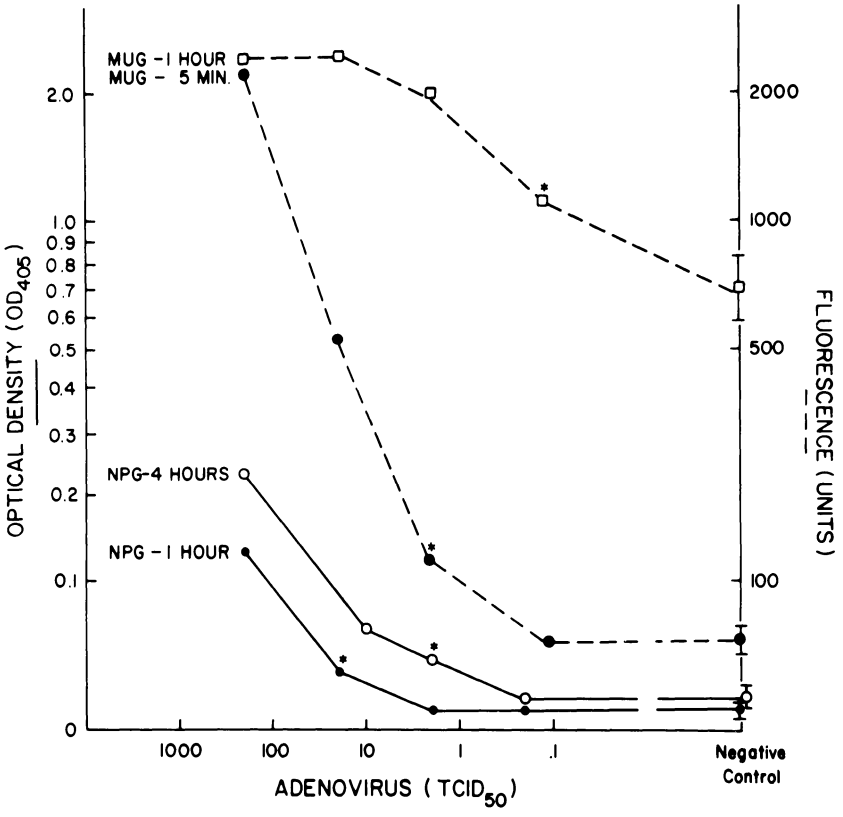

Fig. 7. Comparison of fluorescent and colorimetric substrates for the detection of adenovirus type 29. All assays were performed with rabbit anti-adenovirus bound to the solid phase and monoclonal antibody to adenovirus hexon antigen as a liquid-phase antibody. Goat antibody to mouse IgG labeled with β-galactosidase was used to measure the bound IgG. *Solid lines* indicate the optical density values with nitrophenyl galactoside (*NPG*) the colorimetric substrate. *Dotted lines* indicate the relative fluorescence with the methyl umbelliferyl galactoside (*MUG*). *Vertical bars* indicate one standard deviation of the negative control. *Asterisks* indicate the minimum dilution which will give a fluorescence or optical density value statistically greater than the negative control specimens

dengue virus (reagents supplied by Dr. M. Gentry, Walter Reed Army Institute of Research, Washington D.C.), some of the monoclonal antibodies directed against the antigens could be utilized to formulate assays as sensitive as similar ones with polyclonal antibodies. In the case of adenoviruses (*Cepko* et al. 1981) (reagents supplied by Dr. C. Cepko, Massachusetts Institute of Technology, Cambridge, Massachusetts), we were able to develop enzyme immunoassays which were more sensitive than those in which available polyclonal antibodies were used, especially using fluorescent substrates (Figs. 7, 8). Similarly, other investigators have reported that monoclonal antibodies to hepatitis B surface antigen can be utilized to devise more sensitive assays for the virus (*Hands* 1982). Our data thus suggests that in cases where monoclonal antibodies of sufficient affinity are obtained, enzyme immunoassays which are more sensitive than those using polyclonal antibodies can be formulated, as predicted from the theoretical data in Table 2. Further efforts should thus be directed at devising methods of immunization, fusion, and screening to optimize the recovery of monoclonal antibodies suitable for solid-phase immunoassays.

Monoclonal antibodies have been particularly useful in enzyme immunoassay systems which employ high-energy substrates. These systems are similar in design to standard enzyme immunoassays except that they use substrates which generate radioactive, fluorescent, or chemiluminescent products upon enzyme activation (*Cheetham* and *Dance* 1976; *Delumyea* and *Hartkopf* 1976; *Harris* et al. 1979; *Puget* et al. 1977;

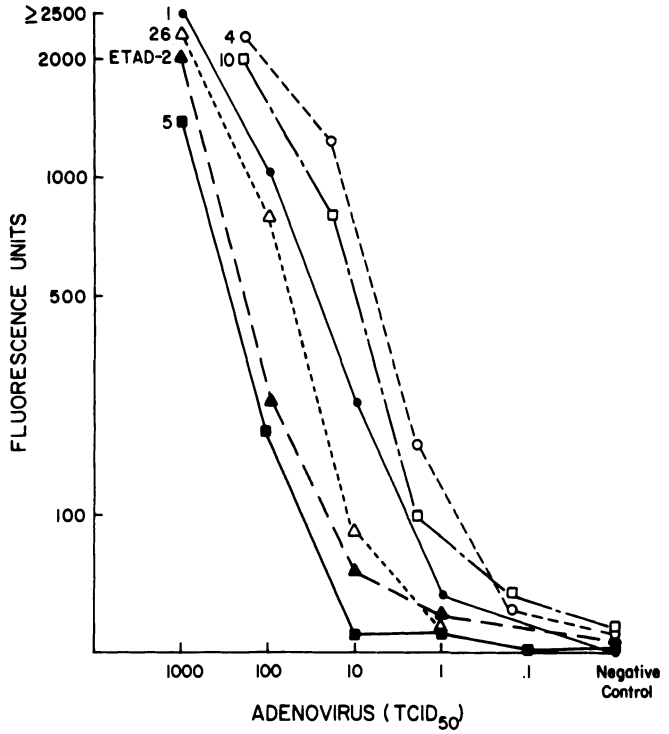

Fig. 8. Fluorescent enzyme immunoassays for different types of adenovirus using monoclonal antibody. The assays were performed as described for Fig. 7. Fluorescence is expressed in arbitrary units. One unit corresponds to approximately 3 pmol 4-methyl umbelliferone. *ETAD = enteric type adenovirus

Rietz and *Guilbault* 1975). Since these materials can be detected at levels 10–1000 times lower than the visible, colored substrates usually used to measure enzyme-substrate reactions, these systems offer the potential for greatly increased assay sensitivity. However, when polyclonal antibodies are employed, this higher degree of sensitivity often cannot be realized due to the occurrence of nonspecific interactions. On the other hand, we have found that monoclonal antibodies can be used to take advantage of this additional sensitivity since nonspecific reactions are much less likely to occur. We have found the most practical high-energy system is one which uses antibodies labeled with β-galactosidase and the fluorescent substrate 4-umbelliferyl galactoside to measure the enzyme-substrate reaction. The availability of simple equipment for the visualization and measurement of fluorescent reactions in small volumes has markedly simplified the use of these substrates. High-energy systems might also compensate for the low affinity of some monoclonal antibodies since these systems allow the recognition of the binding of small amounts of enzyme-labeled antibody in a practical period of time. Enzyme immunoassays using monoclonal antibodies and high-energy substrates might thus provide a very sensitive and practical enzyme immunoassay system.

Efficient diagnostic systems require the identification of a wide range of wild-type antigens in forms in which they might occur in clinical specimens. The question must thus be raised whether monoclonal antibodies can be identified which possess a sufficiently broad range of reactivities to provide useful immunoassay systems. Studies in our laboratory and others have indicated that such monoclonal antibodies

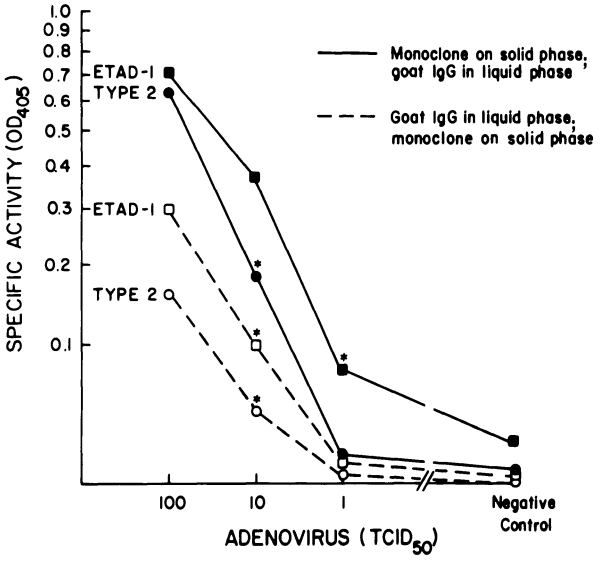

Fig. 9. Comparison of EIA systems for adenovirus. In reactions indicated by a *solid line*, monoclonal antibody to adenovirus hexon antigen was used to coat the solid phase, rabbit anti-adenovirus was used as the liquid-phase antibody, and alkaline phosphatase-labeled anti-rabbit IgG was employed to quantitate binding of rabbit IgG. In reactions indicated by a *dotted line*, the rabbit antibody was used to coat the solid phase and the monoclonal antibody was utilized as the liquid-phase antibody. The reaction was quantitated by the addition of an alkaline phosphatase-labeled anti-mouse IgG. Values indicated by an *asterisk* give the minimum dilution which was statistically greater than the negative control. *ETAD = enteric type adenovirus

can be developed. For example, we have found that the monoclonal antibody prepared by *Cepko* et al. (1981) against the adenovirus common antigen could react with all known types of human adenovirus antigens, including recently identified enteric type adenoviruses (Fig. 9). In addition, this monoclonal antibody could react with viral antigens present in clinical specimens such as stools. This range of reactivity is undoubtedly due to the fact that this antibody was prepared against the hexon antigen, which is shared by all human adenoviruses. Similarly, monoclonal antibody prepared against human rotavirus by Dr. H. Greenberg was found capable of reacting with rotaviruses in all available stool specimens. This range of reactivity was also due to the fact that this antibody reacts with a part of the rotavirus virion thought to be common to all animal and human rotaviruses. Similarly, monoclonal antibodies prepared against dengue virus by Dr. M. Gentry, were capable of reacting with different types of dengue virus (Fig. 10). In addition, monoclonal antibodies prepared against hepatitis B virus by Dr. J. Hands (Massachusetts General Hospital, Boston, Massachusetts) were shown to react with virtually all clinical specimens from patients infected with hepatitis B virus (*Hands* 1982). These findings indicate that although some monoclonal antibodies undoubtedly are too specific to react with a wide range of antigens, monoclonal antibodies can be produced which have sufficiently broad reactivity for use in immunoassay systems. The efficient production of such antibodies depends upon immunization and fusion methods which optimize the possibility of obtaining common antibodies and upon the screening of monoclones to detect monoclonal antibodies with broad-scale reactivities.

In cases in which such clones are difficult to identify, it is often possible to formulate mixtures of pools of monoclonal antibodies capable of reacting with a broad range of viral antigens. This approach has been utilized to prepare reagents

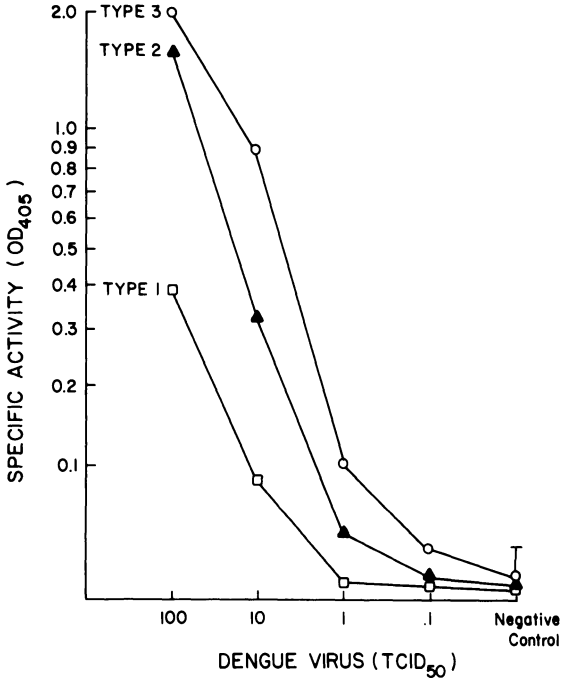

Fig. 10. Enzyme immunoassay for dengue virus using monoclonal antibody. This system utilizes a monoclonal antibody prepared against dengue virus type 2 to coat the solid phase. This monoclonal antibody was shown to have reactivity with all types of dengue virus by immunofluorescence. Human antibody against dengue virus was employed as the liquid-phase antibody, followed by alkaline phosphatase-labeled anti-human IgG. *Vertical bars* indicate one standard deviation of the measurement of the negative controls. *TCID = tissue culture infections dose

capable of reacting with a wide range of herpesviruses and influenza viruses (*Brown* 1982; *Pereira* et al. 1982). Thus, in the cases in which a single, widely reacting monoclonal antibody cannot be derived, pools of monoclonal antibodies might be used to provide practical immunodiagnostic reagents capable of reacting with a wide range of naturally occurring antigens.

7 Conclusions

Monoclonal antibodies have great potential as important tools for immunodiagnosis. It is clear that monoclonal antibodies can be utilized in a wide variety of immunoassay systems in a manner similar to that for polyclonal antibodies, but without the disadvantages inherent in the generation of antibodies in animals. Although the affinity characteristics of all monoclonal antibodies might not be optimal, our data demonstrate that some monoclonal antibodies can be utilized to formulate immunoassay systems which are at least as sensitive as those in which polyclonal antibodies are used. This is especially true if highly sensitive assay systems are employed. In addition, the theoretical consideration that monoclonal antibodies might be too specific for use in immunoassays is not borne out, as evidenced by the fact that monoclonal antibodies can be generated which are capable of reacting with a wide range of antigens from rotaviruses, adenoviruses, hepatitis B virus, herpesviruses, and other viral pathogens. The principal limitation of monoclonal antibody technology continues to be the tediousness of the procedures necessary for generation of the clones, especially if high-affinity, wide-reacting clones are desired. The availability of simple and reliable techniques for producing such clones would markedly expand the scope and practicality of monoclonal antibody use and would bring the advantages inherent in the use of these antibodies to virtually all immunoassay systems.

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High-Resolution Protein Separation and Identification Methods Applicable to Virology**

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1 Introduction

The location, molecular structure, and stage of assembly of viruses and viral components in cells, tissues, and body fluids at various times after infection are important for the understanding of viral diseases. It is equally important to examine in detail the

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morphological, structural, and molecular alterations occurring in native cell constituents after viral infection.

To explore virus-associated changes in detail, separation and analytical systems are required which allow tissues to be disaggregated into cells, the cells in turn to be fractionated into subcellular components, and the isolated components to be resolved into discrete molecular species.

Density gradient centrifugation, electro-optical cell sorting using fluorescent antibodies, and electrophoretic and affinity methods have partially solved the cell separation problem; while zonal centrifugation, preparative free-flow electrophoresis, and affinity techniques have facilitated subcellular fractionation.

The so-called s-ρ methods (*Anderson* et al. 1966), involving a two-dimensional separation based on sedimentation rate (s) in the first dimension and isopycnic banding density (ρ) in the second, led to the discovery of the so-called virus window, which is a region in the s-ρ plot where most virions appear, but which is empty in most plots of non-virus-infected cells and tissues. Fractionation of this type is capable of separating very small amounts of virus from tissues, and the application of the method resulted in the first isolation of hepatitis-associated particles from human plasma in 1964–1965 (*Harris* et al. 1966). Unfortunately, these methods have been used rarely in experimental virology, although they have great potential in the search for viruses that do not initially grow in standard cell lines under normal conditions. The general principles have been combined in continuous-flow-with-banding centrifuges now widely used for large-scale virus vaccine purification (*Anderson* et al. 1969).

The most difficult problem, however, has been to resolve the complex mixture of proteins found in virions, cells, and cell fractions. Hence, the problem has been first to develop, in a routinely usable form, the highest resolution protein separation methods available, and then to apply them in a systematic fashion to both normal and virus-infected cells. This work is in progress, and therefore this is a progress report.

2 Separations Based on Affinity Chromatography

Although affinity columns using antibodies have been widely used for the isolation of specific proteins, their use in resolving more complex mixtures has received rather less attention. Group separations may be performed using immobilized substances which bind to certain sets of proteins, for example, those binding certain nucleotide cofactors, heparin, heme, etc. Of greater interest are separations involving the use of sets of antibodies against sets of protein which either allow these proteins to be immobilized and isolated, or which facilitate the isolation of those not bound. Methods have been developed for systematically producing balanced columns to remove proteins normally present in a mixture (*Anderson* et al. 1975a, b), allowing proteins normally not present (and possibly of viral origin) to pass through. These methods are simple in operation but require lengthy development of suitable immobilized antibody mixtures. It would be extraordinarily useful to have commercially available balanced high-capacity columns for removing normal protein from plasma or tissues. This would facilitate the isolation of those proteins not normally present. Systems for rapid recycling of affinity columns have been developed which allow the same

columns to be used over again hundreds of times automatically (*Anderson* et al. 1975a). The possibility of using very small columns initially and then alternating the use of antibody and antigen columns to scale up gradually to a preparative level has not been fully explored.

3 High-Resolution Two-Dimensional Electrophoresis

The highest resolution analytical system currently available for proteins is two-dimensional electrophoresis in acrylamide gels using isoelectric focusing in the presence of urea and the detergent NP40 in the first dimension and electrophoresis in the presence of sodium dodecyl sulfate (SDS) in the second (*Scheele* 1975; *O'Farrell* 1975). These techniques separate on the basis of charge and mass, which are almost totally unrelated parameters. The resolution of each of these techniques using a suitable test mixture is well over 100 proteins, yielding a theoretical resolution for the two-dimensional system of approximately 10 000 proteins. One of the first problems has been to develop this method into an analytical system which allows large numbers of analyses to be run reproducibly (*Anderson N.G.* and *Anderson N.L.* 1979).

3.1 The ISO-DALT System

A system for casting and running 20–40 isoelectric focusing gels in parallel, and a matching system for casting 20 and running 20 or more slab gels in parallel have been developed (*Anderson N.G.* and *Anderson N.L.*, 1978; *Anderson N.L.* and *Anderson N.G.* 1978). These systems have allowed us to run over 50 000 analyses to date. Additional modifications to improve reproducibility and resolution are now in progress. The end objective is a totally automated analytical system for clinical use.

3.2 Image Analysis and Data Reduction

A very large amount of data is acquired in these analyses. Image data is gathered by scanning dye-stained or silver-stained gels or by scanning autoradiographs or fluorographs. After correcting for film response, or departure from Beer's law in the case of stained images, the pattern is reduced to an array of Gaussian spots, and overlapping spots are resolved (*Taylor* et al. 1980, 1981, 1982). Experimental patterns are stretched constellationally to match standard patterns, and the data is then stored as a spot list with sufficient information attached to each spot to allow the pattern to be reconstructed.

The end result in the TYCHO image analysis and data reduction system, which allows sets of analyses to be compared and differences to be presented as plots of amounts of protein in individual spots in two compared gels; or differences may be indicated by color changes on high-resolution color CRT displays (*Anderson* et al. 1981). A variety of other data reduction systems for two-dimensional gels have also been described (*Bossinger* et al. 1979; *Lemkin* et al. 1979; *Garrels* 1979; *Mariash* et al. 1982). It is not enough to be able to compare a small series of gels, however. One must be able to key into the increasing body of knowledge concerning individual proteins

and protein sets through the images themselves. This is now being done in several different ways.

First, each spot in a reference pattern of a cell or body fluid has a master spot number. By setting the cursor on a spot, that number appears together with (on a separate screen) a menu listing the types of information available (*Anderson* and *Anderson* 1982). Thus one may choose to have all known names for a particular protein, or its intracellular location, cells in which the protein is expressed, molecular mass, or a list of references relating to the protein indicated. This may be done not only for individual proteins, but also for sets of proteins (*Taylor* et al. 1982). For diagnostic purposes, one would want to know which proteins differ in a major way from those found in control preparations, and further, whether the sets of proteins which differ have anything in common. Have they been seen to vary together before? Are they all affected by a know drug? Are they indicative of a particular viral infection? To answer these questions, it is necessary to be able to confer interactively with a very large and constantly incrementing data base. From the point of view of this conference, it is important that the data base include information on virus-encoded proteins, and on cellular proteins altered in amount, distribution, or structure as a result of viral infection.

3.3 Standard Reference Maps

It is important to have reference maps of cells and tissues so that pre- and postinfection patterns can be compared. We have felt that it is important to identify as many proteins on these maps as possible and to determine spot coordinates as accurately as possible. The first problem has been to see if reproducible results can be obtained and whether the level of genetic variability seen in human cells and tissues is so high that identifications cannot be made. With systems for doing large numbers of analysis in parallel, and with careful attention to technique and to reagent quality, high reproducibility can be achieved. The problem of genetic variability has often been overestimated. In practice the level of heterozygosity for a given protein appears to vary greatly with its function, and proteins involved in structure formation appear to be under much greater constraints than those which are apparently free in solution, e.g. cell sap or plasma proteins (*McConkey* 1982). When lymphocytes from unrelated individuals are compared, approximately 1% of the spots on two-dimensional maps are not congruous (*Anderson N.L.*, unpublished data). Identifications may therefore be made with some certainty.

In this laboratory maps have been prepared of human plasma (*Anderson* and *Anderson* 1977, 1979; *Anderson N.L.* et al. 1982; *Anderson* 1981a), lymphocytes (*Anderson* and *Anderson* 1979; *Willard* and *Anderson* 1980, 1981; *Anderson* 1981b, c; *Willard* 1982a, b; *Anderson N.G.* et al. 1982; *Gemmell* and *Anderson* 1982; *Willard* et al. 1982; *Giometti* et al. 1982), muscle (*Giometti* et al. 1979, 1980a, b; *Giometti* and *Anderson* 1981b), hair follicles (*Anderson N.G.* and *Anderson N.L.* 1979), red-cell lysates (*Edwards* et al. 1979, 1981), fibroblasts (*Giometti* and *Anderson* 1981), urinary proteins (*Anderson* et al. 1979a, b; *Tollaksen* and *Anderson* 1980; *Edwards* et al. 1982), seminal plasma (*Edwards* et al. 1981), milk (*Anderson N.G.* et al. 1982), and saliva (*Giometti* and *Anderson* 1979).

3.4 Internal Standards

Two general types of internal standards are required. The first type aims at ultimately yielding numerical values for charge and SDS mass. We have prepared charge standards by sequentially carbamylating proteins such as creatine phosphokinase, carbonic anhydrase, or hemoglobulin subunits (*Anderson* and *Hickman* 1979; *Tollaksen* et al. 1981). When these are added to the initial sample mixture, an even series of spots, each differing by one charge, is produced across the gel, and the longitudinal position of other proteins may be compared with these reference spots in the standard charge train. Molecular mass standards have been prepared from muscle protein extracts (*Giometti* et al. 1980b) and may contain over 80 bands on SDS electrophoresis. The molecular mass standards are added in the agarose used to seal the first dimension gels into position between the second dimension plates.

The second type of internal standard is useful for studies on single proteins or very simple protein mixtures. The protein(s) of interest are run against a background of a known and characterized cell extract of which most proteins have been characterized for charge position (pI) and mass. Dr. Robert Stevenson, Director of the American Type Culture Collection, is arranging to prepare such standard cell preparations as a reference for general use.

4 Protein Identification

It is important to identify as many proteins in gel patterns as possible and to be able to reidentify them definitively in subsequent studies. Identification by position and spot numbers is obviously one method; however, it is insufficient for many of our purposes.

4.1 Identification by Activity

The obvious method for the assignment of an enzyme or other activity to a spot on a two-dimensional gel is to isolate the protein in question by classical methods and run it with and without samples of the starting mixture. (Alternatively, the purified protein may be labeled in a way which does not alter its charge.) If a number of different proteins in a complex mixture are to be identified, this is an extraordinarily laborious approach. In addition, many proteins are found to be partially degraded during isolation, especially if heating is used. Hence, other more convenient methods for identification are required.

When large numbers of gels may be run conveniently, and when enzyme assays may be run quickly and in large numbers, with a centrifugal fast analyzer (*Anderson* 1972) for example, then an alternative and more general approach may be taken. The starting mixture (e.g., an extract from virus-infected cells) is run through a series of separative procedures, and two-dimensional maps are made from all fractions. In parallel, enzyme activities of interest are measured in all fractions. From this data it can readily be determined which spots always correlate with a given activity (and several candidate spots for one activity may be found), and one can also determine

the most logical approach for the isolation of any enzyme (or any given spot). General methods have not previously been available for designing preparative procedures for the isolation of individual enzymes or antigens. This approach provides such a method. So far, it has been applied only to thermal denaturation (*Nance* et al. 1980), where it has proven to be quite useful. Substrates and cofactors may grossly alter the behavior or thermal denaturation temperature of specific enzymes during isolation and thus provide useful information both for identification and to guide isolation. This approach is useful when the objective is the identification of many different proteins in a protein mixture, as is the case with the Human Protein Index Project (*Anderson* and *Anderson* 1982).

4.2 Identification Using Antibodies

When antibodies against one or more proteins in a mixture are available, these may be used to assist in identifications and to solve the problem of whether spots seen in similar positions in different mixtures are indeed related or identical. Antibodies may be used in three general ways. If antibodies with known specificities are available, immunoprecipitates may be prepared or the antibody may be immobilized and used to isolate the antigen. The immunoprecipitates or the purified antigen may then be mapped. Alternatively, the antibodies may be used to remove one spot from the entire mixture, and that spot can then be identified on maps by difference. In the third method, entire two-dimensional patterns may be transferred electrophoretically to nitrocellulose or other immobilizing supports on which they renature sufficiently, in most instances, to bind polyvalent antibodies, which may then be detected in turn with suitably labeled counter antibodies (*Anderson* 1981a). Most monoclonal antibodies studied so far do not react with proteins on nitrocellulose transfers, however. It should be noted that polyvalent antibodies bound to spots on transfers may be used to immobilize an additional layer of nondenatured antigen which may be prelabeled, have been isolated in pure form, or which may be tested for enzymatic activity on the transfer using cytochemical techniques. In addition, very small amounts of specific antibody can be eluted from individual spots on transfers and used for cytological localization studies or for the isolation of small amounts of antigen using micro versions of the CYCLUM system (*Anderson* et al. 1975a).

4.3 Genetic Methods for Identification

Cells or viruses known to lack a particular enzyme, activity, or antigen may be mapped and compared with wild types to find which proteins are missing or altered. In many instances more than one protein is affected by a mutation or deletion, and a clear identification may not be possible. However, candidate spots can be located for further study.

4.4 Peptide Mapping

Single spots from two-dimensional gels may be conveniently cut out, reelectrophoresed through a band of an SDS-resistant protease, and the resulting peptide bands compar-

ed (*Giometti* and *Anderson* 1981a). Although pattern similarity does not prove that the compared proteins are identical, nonmatching patterns rule out identity.

4.5 Amino Acid Sequencing

Several laboratories are developing microsequenators which allow partial sequencing of a protein eluted from a single spot on a two-dimensional gel. This in turn allows the synthesis of a DNA probe with which to isolate the relevant gene. This completes the development of the minimum techniques required to interrelate high-resolution protein mapping and genetic engineering, and it allows, in theory, proteins identified on protein maps to be produced in large amounts for research purposes, for the development of clinical tests, or for replacement therapy.

5 Protein Modification and Processing

Many proteins synthesized for export (for example, cell surface or plasma proteins), many viral proteins, and a fraction of intracellular proteins are modified or processed post-translationally. It is useful to identify the primary translation product, and this may be conveniently done by translating isolated mRNA. Careful pulse-labeling studies using protease inhibitors in the solubilizing media, the use of inhibitors of glycosylation, and neuraminidase digestion of sialated proteins allow the relationships between the primary product and the processed products to be worked out. In addition, labeled primary products may be added to cell extracts and the reactions followed in time. Two-dimensional mapping using both isoelectric focusing to equilibrium and non-equilibrium pH gradient electrophoresis (so-called BASO gels) allow nearly all the components of a mixture to be seen and followed in time (*Willard* et al. 1979), facilitating the identification of intermediates and stages in processing.

6 Organization in Structures

To determine how specific proteins are arranged to form structures, it is essential to determine which proteins are nearest or contact neighbors. By cross-linking neighboring proteins with reagents which can be subsequently dissociated, dimers and multimers may be isolated, dissociated, and then mapped, as has been done with the Rauscher murine leukemia virus envelope glycoprotein (*Zarling* et al. 1980), for example. In addition, the subunits of multimeric proteins may be identified by electrophoresing protein mixtures in one direction in undissociated form, and in the second direction, after dissociation into subunits (*Nagai* et al. 1978). All proteins which were initially monomeric fall on a diagonal in such an analysis; those initially multimeric do not. Hence the term "diagonal electrophoresis".

7 Preparative Scale Counterparts

To make full use of high-resolution protein mapping, preparative methods of equal resolution are required. In some instances, proteins from spots recovered from the

analytical gels can serve for preparative purposes, and sufficient protein recovered to stimulate antibody production. However, there is still a requirement for isolating proteins from minor spots which may not be very antigenic. Intense efforts are now underway in several laboratories to provide high-resolution preparative counterparts of analytical two-dimensional gels (*Edwards* and *Anderson* 1981).

8 Variations of Two-Dimensional Electrophoresis

The two highest resolution analytical methods currently available for polypeptide separation are isoelectric focusing in the presence of urea and a nonionic detergent, and electrophoresis in the presence of SDS. Hence, the combination of these methods yields the highest resolution two-dimensional method and is referred to here as high-resolution two-dimensional gel electrophoresis. Any two other electrophoretic methods may also be combined, and nearly all have been for one purpose or another. For example, a nondenaturing two-dimensional separation may be carried out using nondenaturing isoelectric focusing in one dimension and nondenaturing gradipore electrophoresis in the second. Alternatively, standard gel electrophoresis, which depends on charge-to-mass ratio and to a lesser extent on sieving, may be used in one dimension; or, conventional electrophoresis at two different pHs may be combined. For studies on nearest-neighbor identifications, neighboring proteins may be cross-linked by dissociable reagents and separated in one dimension in cross-linked form by mass using SDS electrophoresis. They may then be separated in the second dimension in SDS after dissociation, as mentioned above. This allows identification of the cross-linked species.

9 Applications of Two-Dimensional Electrophoresis in Virology

The number of proteins encoded by the DNA or RNA of viruses is very small relative to the number of proteins encoded by mammalian host cell DNA. Using postinfection pulse-labeling studies, it is quite simple to sort out virus-encoded proteins from those of the host cell. For many studies on virion protein, especially those of small viruses, the high-resolution systems described here are not essential. Two-dimensional electrophoresis has been applied, however, to the analysis of a number of viral proteins as shown in Table 1 (Note that several different types of two-dimensional electrophoresis are listed).

Clinically, it is of interest to use high-resolution two-dimensional electrophoresis to search for proteins related, either indirectly or directly, to viral diseases in samples which may be available from patients. These may be either of viral origin or may result secondarily from infection (e.g.) by oncogenic viruses which affect host gene expression). Further more, one may be confronted with a previously unknown virus and hence have little idea what to expect, either by way of new physical particles to be seen in s-ρ plots or new spots on two-dimensional gels. In such instances, purely empirical approaches are useful.

Recently, new proteins have been described in the circulating lymphocytes of patients with mononucleosis (*Willard* 1982b), as shown in Fig. 1. In addition, charac-

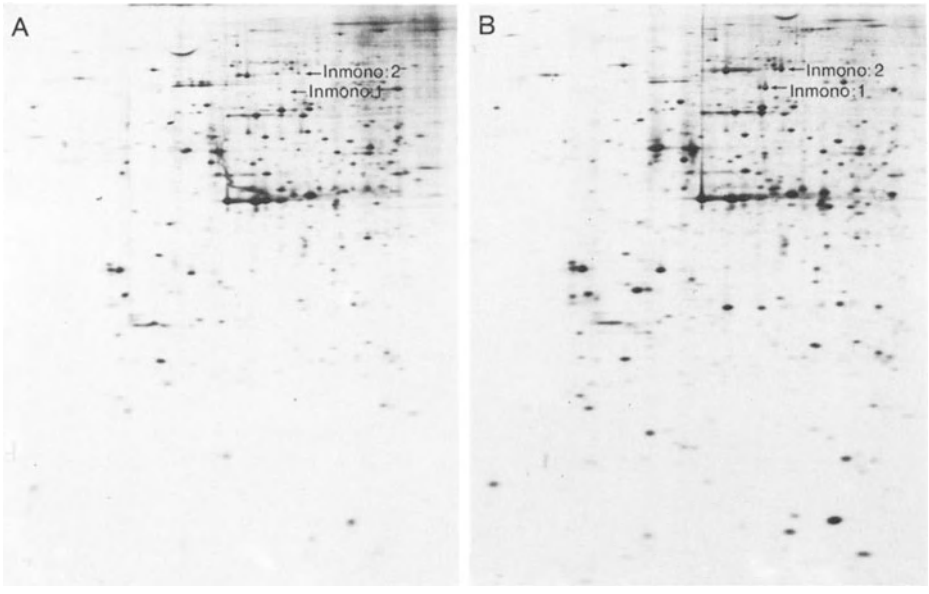
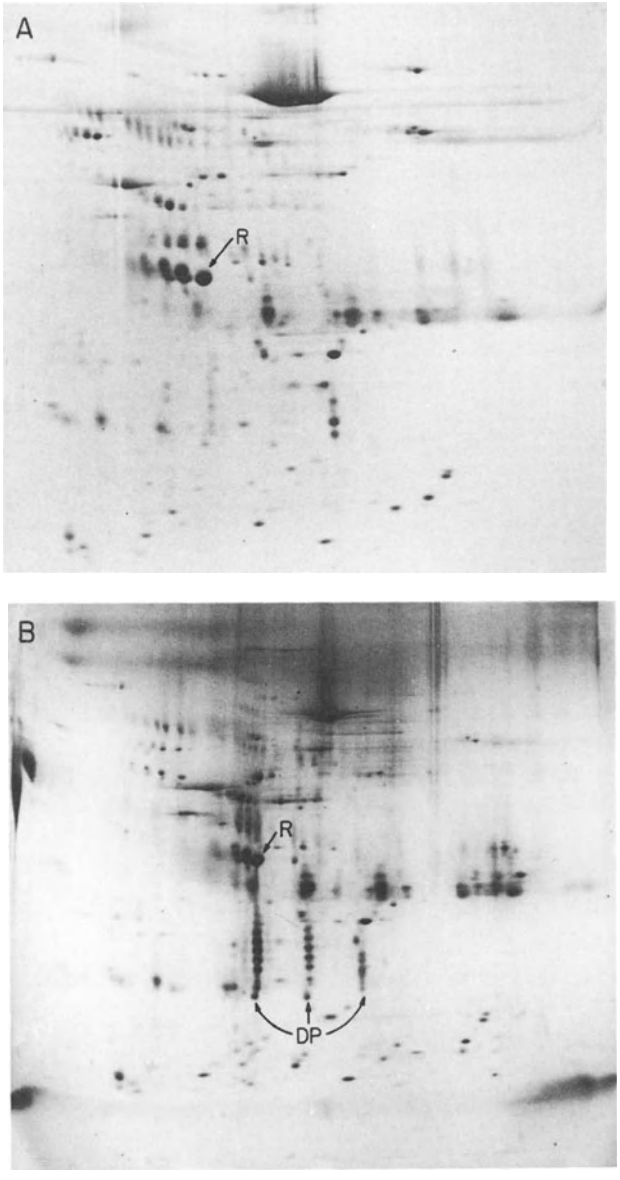


Fig. 1A, B. High-resolution two-dimensional electrophoretic analysis of leukocyte protein pattern changes observed in infectious mononucleosis. Sections of autoradiographs are shown from (A) a healthy 20-year-old female without mononucleosis, and (B) a 20-year-old female mononucleosis patient who was Monospot positive. The *Inmono* (infectious mononucleosis-specific) proteins are identified by number (*Willard* 1982a). The first dimension gels were run with pH 3.5–10 ampholytes. Slab gels were linear gradients of 10%–20% polyacrylamide from the top to the bottom of the gel. The gels are oriented with the basic end of the isoelectric-focusing gel to the right and the acidic end to the left

teristic changes in lymphocyte protein patterns have been observed in maps of lymphocytes from patients with rheumatoid arthritis (*Willard* et al. 1982) (not thought to be of viral origin). Further detailed mapping of human cells and tissues will doubtless turn up many new disease-associated alterations. Most will probably be due to changes in gene expression; however, a few will in all likelihood be due to viral infections. Once a disease-associated alteration is observed, the protein(s) involved may be isolated, antibodies prepared, and epidemiological studies initiated.

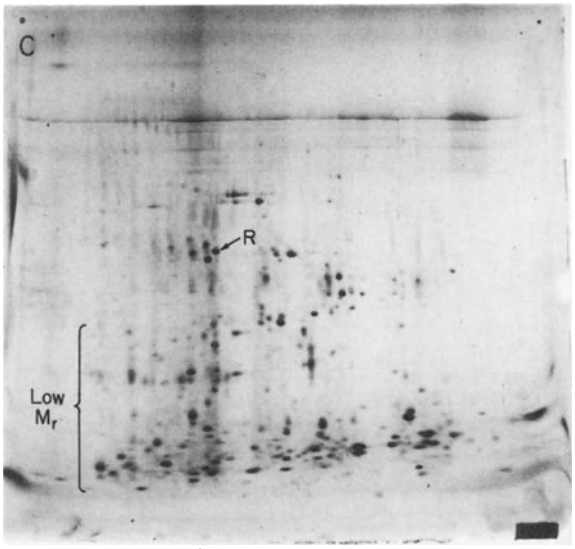
The use of nitrocellulose transfers opens up an additional interesting diagnostic possibility. By comparing patterns stained with pre- and postinfection sera, one may discover which protein in a cell extract is the antigen(s) being reacted to. In previous studies it has been shown that several hundred proteins could be resolved by high-resolution two-dimensional electrophoresis of concentrated human urinary protein samples. The human glomerulus passes proteins below about 50–60 000 Daltons hence, nearly all urinary proteins are below this cutoff point, and antibodies against early proteins of viral infection occur (*Griffiths* et al. 1980). Considering the mass of infected tissue in an average 70-kg man, the fact that infected cells leak viral proteins that some of these proteins are efficiently removed from the circulating blood, and that urine normally contains very little protein, it can be expected that proteins of early virus infection which do reach the circulation (*Griffiths* et al. 1980) will be found in the urine. The first question is simply, is there an increase in low molecular



weight proteins during infection? We have only begun to examine this question and have run urine samples from staff members with fevers of presumed viral origin. The results are shown in Fig. 2 and suggest that a systematic study of urinary proteins in viral infections in which the infective agent is identified would be rewarding. Some of the viral proteins may react on transfers with convalescent sera.

10 Nomenclature of Viral Proteins

If specific viral protein identification is to become more widely used diagnostically, a systematic method for identifying and describing the proteins, analogous to the



▲

◀ Fig. 2. A–C. High-resolution two-dimensional electrophoretic analysis of concentrated human urinary proteins. A) Representative pattern from normal adult male. B) Pattern observed during first day of fever of unidentified cause in an otherwise normal adult male. (a pattern very similar to that in (A) was obtained from this donor before and after illness.) Vertical spot trains marked *DP* are believed to be degradation products of proteins at the top of the train. C) Pattern observed during febrile illness of normal adult female. Note overwhelming increase in low molecular weight (*Mr*) peptides. (Normal patterns were also observed for this donor before and after illness.) Protein marked *R* is a characteristic urinary protein which serves as a landmark for comparing gels A, B and C

system required for the Human Protein Index, will be required. The simplest method would be the virus name, strain, and protein number, with the numbers assigned either in mass order or in chromosome position order. Such combined alphanumeric designators could then be used to interrogate a data base containing sequence and other analytical data as well as information on the source and specificity of both polyvalent and monoclonal antibodies.

11 Conclusions

High-resolution methods for protein separation and analysis allow virus-encoded proteins to be separated and identified and alterations in cellular proteins which may result from virus infection, to be detected. This allows not only a fuller understanding of all steps in virus infection, but the identification and isolation of markers which may be diagnostically useful. As new antiviral drugs become available, and interest increases in *not* using antibodies except when actually needed, it will become important to distinguish bacterial from viral infections early and to provide rapid and specific identifications for each. Tests based on specific viral or bacterial proteins using sensitive immunoassays may solve these problems. The first step is to identify potential indicators, and the techniques described make such identification possible.

Table 1. Two-dimensional electrophoretic analysis of virus-encoded proteins and proteins from virus-infected and virus-transformed cells

Virus	Type of analysis	Method used[a]	Cell or tissue	Reference
Adenoviridae				
Adenovirus 5	CR	B	CV-1 (Green monkey)	*Bosselman* et al. 1978
Adenovirus 5	CR	A (R)	BHK and hamster embryo	*Tuszynski* et al. 1979
Adenovirus 2	VC, CFT	A	KB cells	*Brackmann* et al. 1980
Adenovirus 2	VC	A	KB cells	*Symington* et al. 1981
Adenovirus 12	VCFT	A	Hamster cell lines, rat brain tumor	*Esche* and *Siegman* 1982, *Esche* 1982
Tupaia Virus	VI	A	Isolated virus	*Faissner* et al. 1980
Herpetoviridae				
Epstein-Barr	VTC	A	Burkitt lymphoma, EB^+ lymphoblastoid	*Bachvaroff* et al. 1981
Epstein-Barr	VTC	A	EB-transformed T and B cells	*Altevogt* et al. 1980
Epstein-Barr	VC	A	P3HR-1 Burkitt lymphoma cells	*Roubal* et al. 1981
Herpes type 1	VC	A	KB and BHK cells	*Cohen* et al. 1980
Herpes type 1	VC	A	Various	*Little* et al. 1981
Herpes type 1	VC	A	BHK cells	*Haarr* and *Marsden* 1981
Herpes types 1 and 2	VC	A	Vero cells	*Heilman* et al. 1981
Frog virus 3	VC	A	BHK	*Elliott* et al. 1980
Tupaia herpes	VI	A	Tree shrew tissues	*Faissner* et al. 1982
Papoviridae				
SV40	CR	B	GMK, CV-1	*Bosselman* et al. 1978
SV40, polyoma	VI, VC	A	Mouse embryo, GMK-CV-1	*Ponder* et al. 1977
SV40	VI, VC	A	CV-1	*Milavetz* et al. 1980
SV40, polyoma	VC	A	3T3B-SV40, BHK21 (polyoma)	*Bravo* and *Celis* 1980
SV40	VC	B (M)	GMK CV-1	*Bakayev* and *Nedospasov* 1981
SV40	VC	A	Monkey, mouse, human	*Fanning* et al. 1981
SV40	VC	A	BALB/3T3	*Strand* and *August* 1977

Virus	Type of analysis	Method used[a]	Cell or tissue	Reference
Poxviridae				
Vaccinia	VC, CR	A	Mouseblast carcinoma, EAT mouse L cells	*Beaud* and *Dru* 1980
Vaccinia	VI	A	Mouse L cells	*Tschannen* and *Schafer* 1980
Vaccinia	VC	A, C	KB cells	*Ichihashi* 1981
Monkey poxvirus	VC	A	Cynomolgus monkey kidney cells	*Kilpatrick* and *Rouhandeh* 1981
Coronaviridae				
JHMV and A59V	VC	A	Mouse 17CL-1 and DBT cells	*Bond* et al. 1979
Orthomyxoviridae				
Influenza A	VI	A	Calf kidney cells	*Horisberger* 1980
Influenza A	I	A	Embryonated eggs	*Leavitt* et al. 1979
Influenza A	VI	A	–	*Ulmanen* et al. 1981
Influenza	VI, VC	A	Madin-Darby canine kidney cells	*Privalsky* and *Penhoet* 1978
Influenza	VC	A	Chick embryo fibroblast	*Petri* and *Dimmock* 1981
Paramyxoviridae				
Newcastle disease virus	VI	C	–	*Nagai* et al. 1978
Newcastle disease virus	VI, VC	C	Chick eggs	*Samson* et al. 1980
Newcastle disease virus	VI, VC	A	Chick embryo fibroblasts, chick eggs	*Chambers* and *Samson* 1980
Newcastle disease virus	VC	A	Chick embryo fibroblasts	*Chambers* and *Samson* 1982
Newcastle disease virus	VI	A	Chick eggs	*Smith* and *Hightower* 1981
Newcastle disease virus	VC	A	Chick embryos	*Samson* et al. 1981
Picornaviridae				
Encephalomyocarditis virus	VI	A	Hela cells	*Churchill* and *Radloff* 1981
Poliovirus	VI	A	–	*Hamann* et al. 1977
Poliovirus	VC	A	Hela cells	*Van Dyke* and *Flanegan* 1980
Poliovirus	VC	A	Hela cells	*Wiegers* and *Dernick* 1981
Foot and mouth virus	VC	A	–	*McCahon* 1981
Foot and mouth virus	VI, VC	A	BHK cells	*King*, this volume, p 219

Table 1. (continued)

Virus	Type of analysis	Method used[a]	Cell or tissue	Reference
Rhabdoviridae				
Rabies	CR	A	BHK cells	*Naito* and *Matsumoto* 1978
Vesicular stomatitis	CR	B	Mouse L cells	*Marvaldi* and *Lucas-Lenard* 1977
Vesicular stomatitis	VI	C	BHK-21 cells	*Dubovi* and *Wagner* 1977
Vesicular stomatitis	CR	B	CV-1 cells	*Bosselman* et al. 1978
Vesicular stomatitis	VI	C	BHK-21 cells	*Muds* and *Swanson* 1978
Vesicular stomatitis	CR	B	Mouse L cells	*Jaye* et al. 1980
Vesicular stomatitis	VI	A	–	*Kingsford* et al. 1980
Vesicular stomatitis	VI, CR	C	BHK	*Thimmig* et al. 1980
Vesicular stomatitis	CR	A	Human fibroblasts	*Weil* et al. 1980
Vesicular stomatitis	VC	A	Chinese hamster lung V-79	*Maack* and *Penhoet* 1980
Vesicular stomatitis	VI	A	–	*Hsu* and *Kingsbury* 1982
Retroviridae				
Friend Leukemia	CR	A	Friend erythroleukemic cells (FrC18)	*Reeves* and *Cserjesi* 1979
Friend Leukemia	VI	C	Eveline cells	*Schneider* et al. 1980
B-tropic WN1802B	CR	A	Mouse embryo 10T 1/2-cl 8 cells	*Yoshikura* et al. 1982
Maloney murine sarcoma and murine leukemic virus	VI	A	BALB/3T3 fibroblasts	*Forchhammer* and *Turnock* 1978
Murine leukemia virus	VI	C	Mouse 60A lymphoblastic cell line	*Takemoto* et al. 1978
Murine leukemia virus	VC, CR	A	Maloney mouse lymphoma	*Fox* and *Weissman* 1979
Mouse mammary tumor	VI	A	Mm 5 mt/cl cells	*Nusse* et al. 1980
Mouse mammary tumor	VI	C	Mm 5 mt/cl cells	*Racevskis* and *Sarkar* 1980
Kirsten murine sarcoma, Maloney sarcoma virus	C, CR	A	NIH/3T3 and BALB/3T3 cells	*Strand* and *August* 1977, 1978
Gross murine leukemia	VC	A	C57 BL/6, AKR, MOPC–21	*Ledbetter* 1979
Radiation-induced leukemia virus	CR	A	Mouse tumor cells	*Meruelo* et al. 1978
Rauscher leukemia	VC, CI	A	JLS-V16 mouse embryo and JLS-V5 spleen	*Karshin* et al. 1977

Virus	Type of analysis	Method used[a]	Cell or tissue	Reference
Retroviridae				
Rous sarcoma virus	CFT	A	Chick embryo fibroblasts	*Kamine* et al. 1978
Rauscher leukemia	CR	A	Mouse JLSV5	*DeLey* et al. 1979
Rauscher leukemia	VC, CR	C	C57BL/6 mouse leukemia cells	*Zarling* et al. 1980
Avian Rous sarcoma	CR	A	Chick embryo fibroblasts	*Isaka* et al. 1978
Rous sarcoma	CR	A	Chick embryo fibroblasts	*Shiu* and *Pastan* 1979
Rous sarcoma	CR	A	Chick embryo fibroblasts	*Radke* and *Martin* 1979, *Radke* et al. 1980
Rous sarcoma	CR	A	Rat myoblast-mouse fibroblast hybrids	*Brzeski* et al. 1980
Avian sarcoma and murine sarcoma	CR	A	Indian muntjac cells	*Yuasa* et al. 1980
Rous sarcoma	CR	A	Chick embryo fibroblasts	*Laszlo* et al. 1981
Rous sarcoma	CR	A	Chick embryo fibroblasts	*Kobayashi* et al. 1981
Rous sarcoma	CR	A	Chick embryo fibroblasts	*Hendricks* and *Weintraub* 1981
Rous sarcoma	CR, VC	A	Chick embryo fibroblasts	*Gilmore* et al. 1982
Togaviridae				
Sindbis virus	CR	A	Aedes albopictus cells	*Eaton* 1982
Semliki Forest	VI, VC	C	BHK-21	*Richardson* and *Vance* 1978

Abbreviations: VI, isolated virions analyzed; VC, virus-encoded proteins identified in cell extracts; VCFT, analysis of peptides produced by cell-free translation of viral messenger RNA; VTC, virus-transformed cells mapped; and CR, cellular response to virus infection examined, e.g., effect of synthesis of cell proteins
A, isoelectric focusing in urea and NP-40 (either equilibrium or nonequilibrium) in the first dimension and electrophoresis in SDS in the second, according to *O'Farrell* (1975) and *Scheele* (1975); B, electrophoresis in acid urea in the first dimension and SDS in the second for ribosomal subunit analysis; and C, electrophoresis in SDS under nonreducing conditions in the first dimension and in SDS under reducing conditions in the second dimension to study cross-linking of nearest neighbors. R indicates dimensions are reversed; M indicates method has been modified

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Electrofocusing Structural and Induced Proteins of Aphthovirus

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1 Introduction: What is Electrofocusing?

Electrofocusing separates ampholytes such as proteins according to their isoelectric point. Different versions of the technique have been described under various unwieldy names such as isoelectric focusing or non-equilibrium pH-gradient electrophoresis, but we prefer to apply the term electrofocusing to any type of pH-gradient electrophoresis. In this paper we are concerned specifically with electrofocusing in polyacrylamide gels containing 9*M* urea. Under these conditions the protein subunits that make up biological structures can be separated as individual polypeptides with very high resolution.

Electrofocusing has been used surprisingly little in the field of virology and hardly at all as a diagnostic method. In this paper we shall review what kinds of information can be obtained by electrofocusing virus polypeptides and, in particular, we shall demonstrate the potential of the technique in virus epidemiology. The various applications will be illustrated by reference to our studies on aphthovirus, the causative agent of foot-and-mouth disease.

2 Electrofocusing the Polypeptides of a Picornavirus

Figure 1 contrasts a polypeptide pattern obtained by electrophoresis in a sodium dodecyl sulphate (SDS)-polyacrylamide gel with an analysis of the same material by

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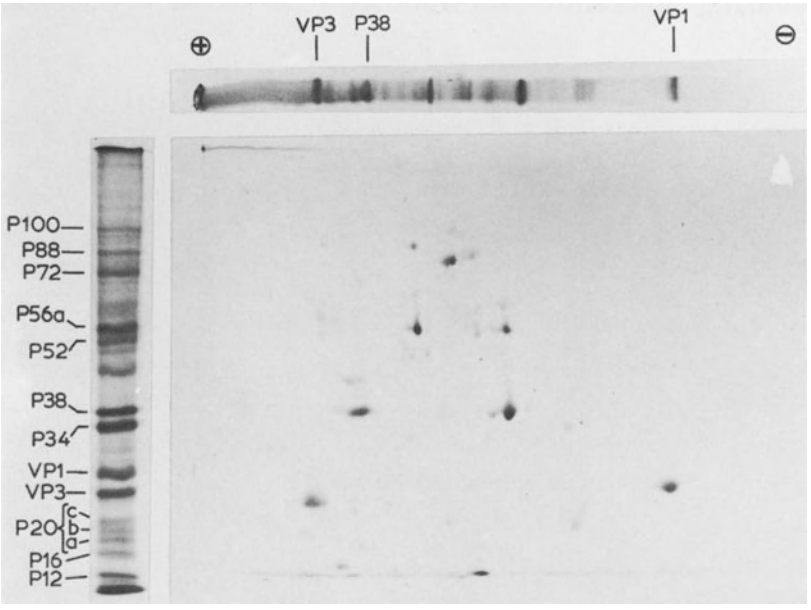

Fig. 1. Two-dimensional electrophoresis of aphthovirus-induced polypeptides. A cytoplasmic extract prepared from infected cells, labelled for 10 min with [^{35}S]methionine, was subjected to electrofocusing in the horizontal dimension in a gel containing pH 3.5–10 Ampholines, followed by electrophoresis in the vertical dimension in a 10% SDS polyacrylamide slab gel. One-dimensional electropherograms of the same material are also shown. The origin of electrophoresis is at the *top left*. (From *King* et al. 1982)

electrofocusing, both individually and in the form of a two-dimensional analysis in which the two techniques are combined (*O'Farrell* 1975). Under the conditions of labelling used, all the known aphthovirus-induced polypeptides except VP2 and VP4 are visualised. To optimise resolution of different polypeptides, conditions can be varied with regard to pH range, direction of electrofocusing or inclusion of non-ionic detergent (see figure legends for details). Aphthovirus polypeptides, like those of other viruses, were originally defined by their migration rates in SDS gels. The two-dimensional pattern enables us to relate the two one-dimensional patterns to each other and thus to identify the polypeptides seen in electrofocusing gels.

Virus polypeptides are seen in Figs. 1 and 3 against a background of cellular proteins. Visualisation of the virus components relies on the ability of the virus to inhibit host protein synthesis during the labelling period. Alternatively, virus-specific proteins can be selected with antiserum and precipitated with protein A-bearing *Staphylococcus aureus* (*Harris* et al. 1981); we routinely use this refinement when examining field isolates of aphthovirus.

Figure 2 shows the coding arrangement of polypeptides along the aphthovirus genome. Picornavirus polypeptides are synthesized from a single initiation site near the 5′-end of the RNA. Mature polypeptides are generated from large precursors by a series of proteolytic cleavages. The relationship between precursors and products on two-dimensional gels is governed by the following rules. In the vertical (SDS)

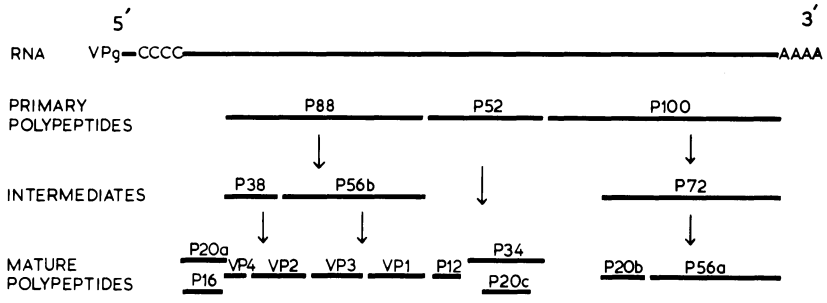

Fig. 2. Biochemical map of aphthovirus polypeptides. Bases on *Sangar* (1979); other sources, *Saunders* and *King* (1982), *K. Saunders*, personal communication

dimension precursors migrate more slowly than their cleavage products. By contrast, the position of a protein in a pH gradient is determined by isoelectric point, and hence in the horizontal (electrofocusing) dimension the position of a precursor approximates to that of the weighted mean of its constituent parts.

Picornaviruses make ideal subjects for electrofocusing for the following reasons. First, all the virus-induced polypeptides can be focused into sharp, usually single, bands. Second, since picornaviruses produce only a single species of polycistronic messenger RNA, their genes are conveniently expressed in equimolar amounts. Third, the number of mature polypeptides is small enough to produce an electrofocusing pattern that can readily be compared in one dimension alongside those of other strains. Finally, picornavirus polypeptides are not glycosylated or phosphorylated, at least not to a significant extent. This greatly simplifies the interpretation of data, since changes in isoelectric point must be a direct manifestation of alterations in the polypeptide-coding sequences. Obviously, electrofocusing can be used with other viruses, a good example being the separation of over 200 different polypeptides induced by herpes simplex virus (*Haarr* and *Marsden* 1981); however, aphthoviruses, being far simpler, make a good starting point for exploring possible uses of electrofocusing.

3 What Can Isoelectric Points Tell Us?

The isoelectric point of a protein is determined by the individual p*K*s of all its ionizable groups. A mutation that substitutes a charged amino acid residue for an uncharged one, or vice versa, will change the isoelectric point of the protein. An example is shown in Fig. 3. The charge change in polypeptide P38 can also be seen in its 88-kilodalton precursor, proving that a mutation affecting one amino acid residue out of as many as 800 can readily be detected by electrofocusing. Not all mutations cause charge changes, but according to the genetic code approximately one-third of all the possible nucleotide substitutions that alter codon usage do lead to a change of charge (*Singer* et al. 1978) and should therefore be detectable. In favourable instances it is even possible to deduce the type of amino acid residue involved (*King* and *Newman* 1980). This gives us a simple yet sensitive probe for polypeptide sequence. There are many ways in which this basic information can be applied. Below are listed briefly somes areas of virology in which electrofocusing has been applied, some of which are described in more detail.

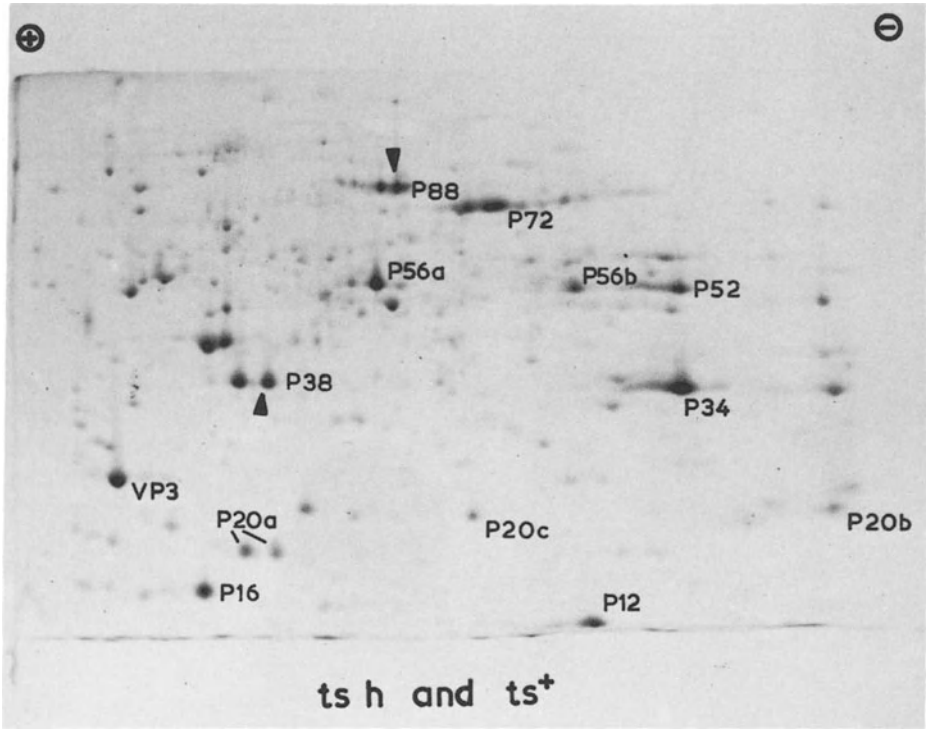

Fig. 3. Two-dimensional electrophoresis of polypeptides induced by a mixture of mutant (*ts h*) and wild-type (ts^+) aphthoviruses. Cells were infected with individual viruses, and extracts were mixed before electrophoresis. Mutant polypeptides are *arrowed*. Electrofocusing was in a gel containing a 1:1 mixture of pH 5–7 and pH 7–9 Ampholines, as described previously (*King* et al. 1982a)

1. Physical mapping of mutations. The locations of conditional-lethal mutations in the aphthovirus genome are described in Sect. 4.

2. Precursor-product relationships. Mutations affecting mature aphthovirus polypeptides are always carried by their precursors as well (see, e.g., Fig. 3). The coding positions of polypeptides P20b and P20c in Fig. 2 were assigned on the evidence of their covariation with P72 and P34, respectively (*Saunders* and *King* 1982; unpublished observations).

3. Genetic recombination in aphthovirus. We have used charge-change mutations as genetic markers for studying recombination between mutants of the same strain (*King* et al. 1982a). The more recent demonstration of recombination between different subtypes of aphthovirus (in which electrofocusing also played a major role) raises the possibility that recombination may be important in the evolution of aphthoviruses.

4. Molecular weight. If the amino acid composition of a protein is known, its molecular weight can be determined by using electrofocusing to count the number of lysine residues that are modified during controlled carbamylation. By combining the electrofocusing data on VP2 and VP3 of aphthovirus (*King* and *Newman* 1980) with amino

acid compositions reported by *Adam* and *Strohmaier* (1974), we calculate molecular weights of 25 000 and 23 000, respectively, which are close to estimates based on sequencing (*Boothroyd* et al. 1982).

5. Evolution. Variation in protein charge among naturally occurring strains has provided an insight into structural and functional constraints that limit evolutionary change.

6. Strain identification. Mutations affecting protein charge have been used as epidemiological markers.

7. Virus classification. The prospects and problems of linking electrofocusing data with serology are considered in Sect. 7.

8. Peptide fingerprinting. This useful variation of the technique is described in Sect. 8.

9. Secondary modification of proteins. Among viruses other than picornaviruses electrofocusing has been used mainly for studying modification of proteins (*Klein* et al. 1979; *Kohama* et al. 1981; *Lanford* and *Butel* 1980; *Nusse* et al. 1980).

4 Physical Mapping of Mutations

We have examined many aphthovirus mutants for alterations in their polypeptides. Of these mutants, 70 were temperature sensitive (*ts*) and had been arranged to form a genetic recombination map shown in Fig. 5. The map also features a guanidine-resistance (*gr*) mutation. We were primarily interested in finding out what relationship, if any, the genetic map bears to the physical genome. However, the results are also of wider interest in that they demonstrate the tendency of mutations to arise in specific genes. Virus-induced polypeptides were compared by one-dimensional electrofocusing. This version of the technique allows the four coat proteins, VP1, VP2, VP3 and VP4, and the major non-structural polypeptides, P34 and P56a, to be

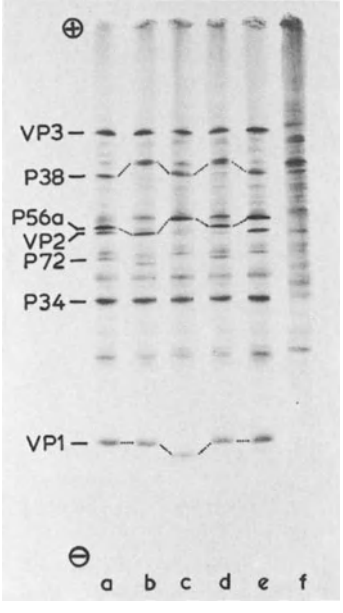

Fig. 4. The covariant reversion test. One-dimensional electrofocusing of polypeptides induced by (*a*) ts^+; (*b*) ts 22; (*c*) ts 40; (*d*) a revertant of ts 22, ts 22 R, able to grow at the non-permissive temperature; (*e*) a revertant of ts 40, ts 40R. Gel (*f*) shows the pattern obtained from uninfected BHK cells. *Dotted lines* between adjacent samples indicate charge differences affecting P38, P56a and VP1; for clarity, differences in VP2, which covaries with P38, and in P72, which covaries with P56a (see Fig. 2), are not indicated. (*King* et al. 1982a)

resolved. In this way approximately 75% of the genome was screened for charge-change mutations.

Spontaneous ts^+ revertants of any *ts* mutant found to have an altered polypeptide were examined in order to test whether a single mutation was responsible for both temperature sensitivity and the charge change. The application of this test, the "co-variant reversion test", is illustrated in Fig. 4. These mutants are chemically induced and each exhibitis charge changes in two independently coded polypeptides; *ts* 22 is altered in VP2 and P56a and *ts* 40 in VP1 and P56a. It can be seen that in their respective revertants one of the altered polypeptides, but not the other, has reverted to wild-type. One this basis the *ts* lesion of *ts* 22 was assigned to the RNA polymerase gene, P56a, a conclusion subsequently confirmed by enzymological studies (*Lowe* et al. 1981), whereas the *ts* lesion of *ts* 40 was assigned to the structural polypeptide, VP1.

All the *ts* mutations of the genetic map have been screened in this way and the results are summarized at the bottom of Fig. 5. Twenty *ts* mutations were found to covary with coat protein alterations (*King* et al. 1980), all of these having map loci to the left of *gr*. Two *ts* mutations were located in the P56a gene, both having loci at the far right of the genetic map. However, none of the 70 *ts* mutations was associated with an alteration in P34. This suggests that *ts* mutations arise most frequently in the coat protein genes, less frequently in the polymerase gene, and rarely if ever in the P34 gene. We shall see in Sect. 7 of this review that the genome distribution of mutations occurring during evolution in the field follows a similar pattern.

Locating the single *gr* mutation of the recombination map presented us with the problem that although the mutation was associated with a charge change in P34, it was not possible to confirm that location owing to the difficulty of isolating sensitive revertants of a resistant mutant. However, other spontaneous *gr* mutants have also been found to induce the synthesis of an altered P34 (*Saunders* and *King* 1982), and it appears that *gr* mutations, unlike *ts* mutations, are confined exclusively to the P34 gene.

Three main conclusions may be drawn from Fig. 5, of which the last two are particularly relevant to the study of virus variation. First, comparison with Fig. 2

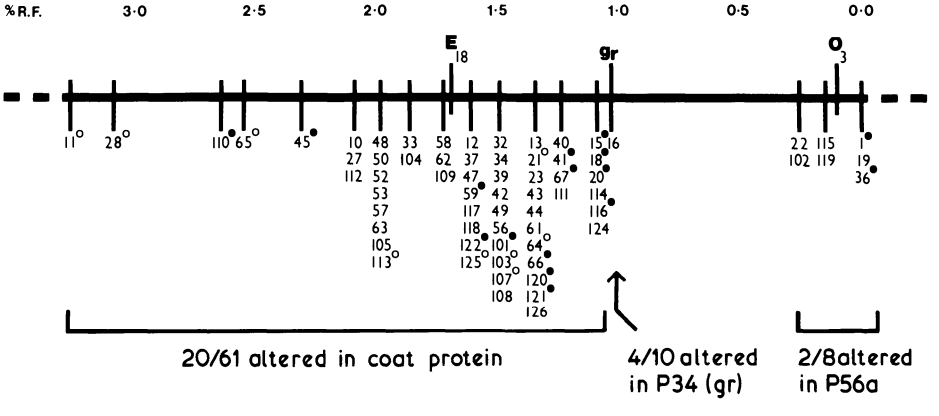

Fig. 5. The physical distribution of mutations of the aphthovirus genetic map as determined by electrofocusing. All mutations are temperature-sensitive except for those designated "*gr*"

shows that map loci are correlated with physical coding position, the genetic recombination map being orientated such that its left-hand end represents the 5′-end of the RNA genome. Second, approximately one-third (26/80) of the mutations involved changes of polypeptide charge. This detection rate is consistent with theoretical expectations (Sect. 3) and apparently applies to both *ts* and *gr* mutations and to each of the three regions of the genome shown in Fig. 5. These findings demonstrate the remarkable sensitivity of electrofocusing to genetic change in viruses, a sensitivity comparable with that of two-dimensional T_1 RNase fingerprinting of the viral RNA. Third, the distribution of mutations in the aphthovirus genome is highly non-random and is characteristic of the type of mutation.

5 Virus Variation: Capsid Polypeptides Evolve at Different Rates

At a molecular level evolution consists of an accumulation of point mutations, which will be revealed as a series of discrete electrofocusing shifts. The rate of evolutionary change is determined primarily by the tolerance of proteins to alterations in amino acid sequence (*Kimura* 1976). In a virus capsid the most severe constraints would be expected to apply to internal protein subunits, whereas proteins on the surface would be expected to tolerate a higher frequency of amino acid substitutions, especially of substitutions involving charge changes.

Among field isolates we have observed greater variation in some capsid polypeptides than in others. Thus, within a set of eight type 0 strains isolated from different parts of the world at different times, the isoelectric point of VP2 was completely conserved, whereas there appeared to be no conservation of VP1 or VP3 (*McCahon* 1981; *McCahon* et al. 1977). More recent work has confirmed the marked conservation of VP2 in this serotype, although a few exceptions have been found (see Fig. 7). The small acidic polypeptide, VP4, was also conserved. Since VP1, VP2 and VP3 have similar sizes, differences in variability presumably reflect different locations in the capsid.

Figure 6 illustrates some results taken from a survey of strains belonging to another aphthovirus serotype, SAT 2. At first sight the variety of protein patterns is bewildering. However, certain evolutionary trends can be discerned and these appear to differ from those of type 0 viruses. As before, VP4 is almost completely conserved and VP1 (not shown) extremely variable, but in these African viruses, VP2 appears to be more variable than VP3. Thus, a set of three VP3 bands, indicated by arrows in Fig. 6, are common to 14 of the 21 isolates, including one isolated more than 30 years ago. By contrast, there is no evidence of conservation of VP2 apart from five viruses from Botswana and Zambia shown in the upper part of Fig. 7. Some isolates, e.g., Botswana B17/69, are clearly mixtures.

Type 0 and type SAT 2 are representatives of the two main phylogenetic lines of aphthoviruses (*Robson* et al. 1977). Why do their capsid proteins evolve differently? The difference is not due to confusion between the identities of VP2 and VP3, and we conclude that VP2 is relatively more tolerant to change in type SAT 2 than in type 0 because VP2 has a more exposed location in the SAT 2 capsid. The fact that VP1 is highly variable in both serotypes conforms with its known surface location (*Rowlands* et al. 1975) and antigenicity (*Strohmaier* et al. 1982).

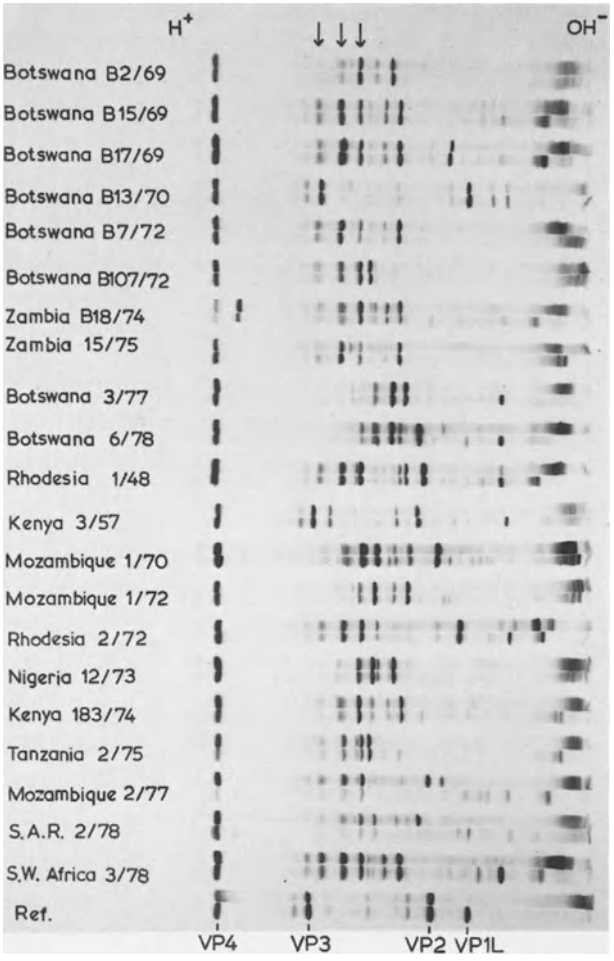

Fig. 6. Comparison of ^{35}S-labelled structural polypeptides of 21 aphthoviruses belonging to serotype South African Territories 2 (SAT 2). Isolates are identified by World Reference Laboratory reference markers, the last two digits giving the year. Strain O_1 Pacheco is included as a reference at the bottom of the figure. Electrofocusing was as for method (ii) of *King* and *Newman* (1980). Under these conditions VP4 is focused, but VP1 stays at the origin at the right hand end. (The VP1 polypeptides were compared in another set of gels which is not shown.) The gels are in pairs, the *upper* showing an analysis of untreated virus, and the *lower*, of trypsin-treated virus. VP1 was identified by sensitivity to trypsin, the large tryptic fragment being VP1L, and VP2 by its relative deficiency in natural empty particles (not shown) as described previously (*King* and *Newman* 1980). In all strains tested VP1, VP2, VP3 and VP4 electrofocused in order of increasing acidity. *Arrows* indicate conserved VP3 bands

Mutations must occur at a similar frequency in all the aphthovirus genes. The difference between a variable and a conserved protein is that mutations affecting the latter are more likely to be selectively disadvantageous and thus be eliminated. This process is illustrated by the most conserved of the structural polypeptides, VP4. Although the isoelectric point of VP4 is the same in all seven serotypes of aphthovirus,

as the type 0 reference in Fig. 6 demonstrates, individual exceptions to this rule have been found among members of three different serotypes including SAT 2 (see Zambia B18/74, Fig. 6). Such mutations must have arisen independently, and each is presumably destined to revert or die out.

6 Strain Identification

Evolution consists of a process of gradual sequence divergence. In order to track down the source of an outbreak, it is desirable to be able to detect the smallest possible difference between viruses, so that one can, in evolutionary terms, follow the trail while it is still hot. The sensitivity to mutation and the ease and precision with which polypeptide patterns can be compared make electrofocusing a uniquely powerful technique in epidemiology. Fig. 7 records a preliminary study of aphthoviruses isolated during the disastrous foot-and-mouth disease epizootic in Britain in 1967–1968. That outbreak is believed to have been caused by a single introduction of virus. Yet the polypeptide patterns reveal several different forms of virus which were presumably generated during passage through the susceptible animal population. This rapid rate of change is explained by the very high mutation frequencies that are characteristic of RNA viruses (*Holland* et al. 1982).

During the period that we have been using electrofocusing, there have been two outbreaks of foot-and-mouth disease among British livestock, the first in Jersey and the Isle of Wight in March 1981 and the second more recently among cattle that had been exported to Libya and were suspected of having carried the disease from Britain. The application of electrofocusing to these two outbreaks is illustrated by Figs. 8 and 9, respectively.

The first outbreak started in Brittany (France) and spread apparently on the wind to Normandy (France), Jersey (U.K.) and the Isle of Wight (U.K.), all four geographical locations lying in an approximate straight line (*Donaldson* et al. 1982). As expected, most of the viruses isolated from these outbreaks produced the same

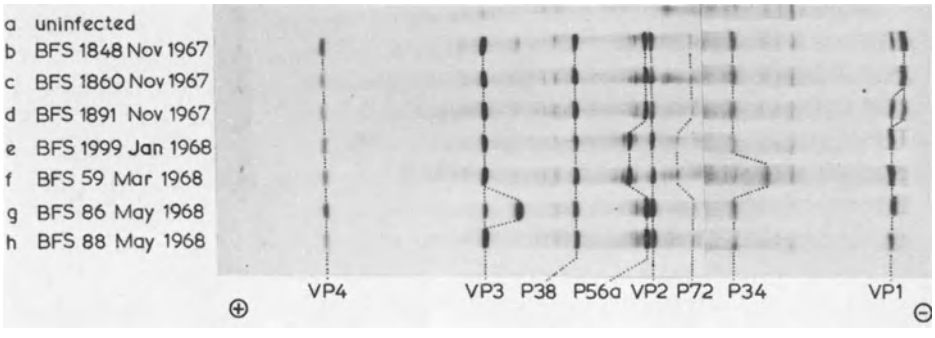

Fig. 7. Comparison of the induced polypeptides of seven British Field Strains (*BFS*) isolated during the 1967–1968 epizootic of foot-and-mouth disease. Virus-specific polypeptides were purified by precipitation with convalescent antiserum. The figure is composed from parts of two sets of electrofocusing gels run with opposite polarity and joined so that redundant information at the origins of the gels is omitted. By this means both VP1 and VP4 can be studied (*King* et al. 1981)

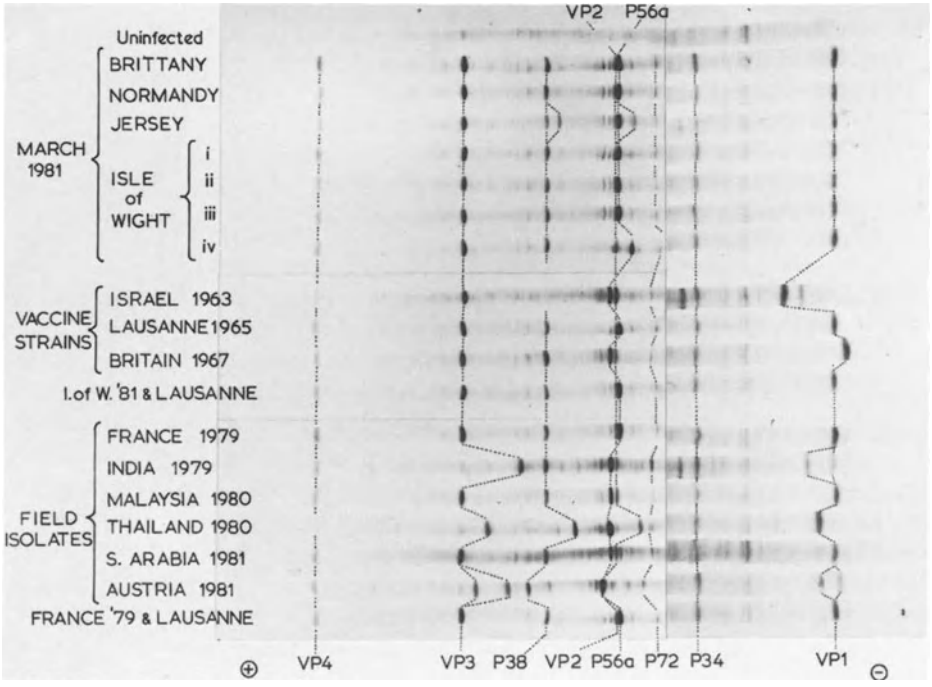

Fig. 8. Identification of the virus strain responsible for the outbreaks of foot-and-mouth disease in France and the United Kingdom in March 1981. Methods were as for Fig. 7. (*King* et al. 1981)

polypeptide pattern (Fig. 8). Where did the initial infection in Brittany come from? Other type O viruses were examined as possible sources. These included three strains isolated many years ago that are currently used in the manufacture of vaccines, and six recent field isolates. Only two of the resulting patterns, 'Lausanne 1965' and 'France 1979', match that of the March 1981 virus exactly (Fig. 8). These relationships were confirmed by RNase T_1 fingerprinting of the virual RNA (*King* et al. 1981). Since the disease is not endemic in France, we conclude that the outbreaks there in 1979 and 1981 were started independently by infection with the Lausanne strain. The significance of this lies in the date of the strain, viz. 1965. We analysed several stocks of the 'Lausanne' strain, one of which had been in our repository at the World Reference Laboratory for 13 years. All gave exactly the same pattern. In view of the rapid rate of change that we see in viruses in the field, it seems inconceivable that the strain could have survived unaltered from 1965 to 1979. The virus must have been, literally, in the deep freeze. We conjecture that it got into susceptible livestock in Brittany as a contaminated or incompletely inactivated vaccine. In principle it should be possible to identify the source more precisely, since stocks of the same strain maintained independently in different laboratories at our Institute have been found in some cases to give slightly different electrofocusing patterns.

In the second example (Fig. 9) viruses isolated from British cattle after arrival in Libya ('Libya 1982') were compared with viruses isolated the previous year in Libya and the United Kingdom (U.K.). Whereas the 1982 viruses are very similar to the 1981 Libyan viruses, differing in just one polypeptide (P34), they differ from the U.K.

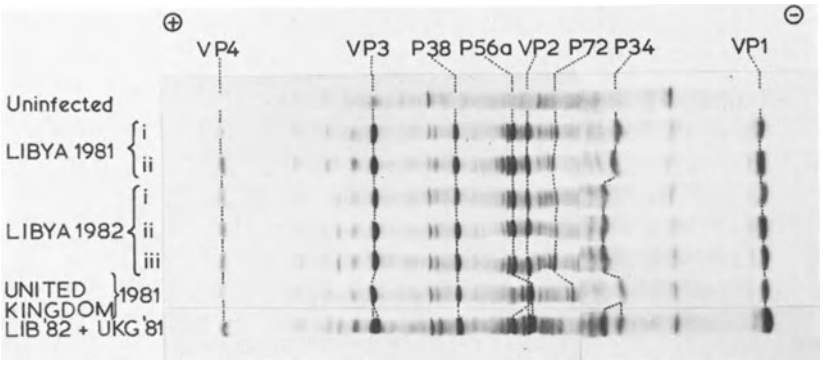

Fig. 9. Did British cattle carry their virus to Libya

strain in three independently coded polypeptides, P34, P56a and, as the mixture in Fig. 9 confirms, VP3. We conclude that the cattle were infected with an indigenous Libyan virus and not with the U.K. strain. Again, the same conclusion was reached independently by RNA fingerprinting (*B.O. Underwood*, personal communication).

In order to produce a polypeptide pattern a virus must be passaged in tissue culture, so running the risk of introducing a mutation. This raises the possibility that some of the differences attributed to evolution may be artifacts. Fortunately, the frequency of charge-change mutations seems to be low. No variant polypeptides were observed in a total of 88 progeny of genetic recombination experiments (*King* et al. 1982a, b). However, these viruses were plaque purified and were well adapted to growth in BHK cells. The possibility remains that the proportions of different virus species in an uncloned field isolate might change during passage in tissue culture. It may be significant that we had difficulty recovering virus from the two variant forms of the March 1981 virus, 'Jersey' and 'Isle of Wight' viruses (Fig. 8). These uncertainties are minimised by using consistent tissue culture practice and if necessary, by examining independent isolates of the same strain, as shown in Figs. 8 and 9. It should be remembered that this problem also applies to other biochemical techniques sensitive to point mutations.

7 Classification

Aphthoviridae are classified into seven serotypes on the basis of cross-protection tests. Within each serotype different strains cross-protect with widely varying degrees of efficiency, and attempts have been made to group them into subtypes using other kinds of serological test. Behind all these attempts at classification lies the need for a reliable method of predicting how effectively immunization with one virus strain will protect against challenge by another. Obviously, viruses having identical or very similar electrofocusing patterns will be related serologically, but the question we are asking here is whether you can use electrofocusing to predict which viruses will *not* cross-protect.

No systematic comparison of electrofocusing patterns with serological properties has yet been undertaken, although we have observed several cases in which a marked serological difference within a group of viruses was associated with multiple polypeptide differences. An example is the detailed study of strains belonging to subtypes A5, A12 and A22 by *Robson* et al. (1979). In addition, several viruses in Fig. 8 have been compared serologically (*M. Lombard* and *A. Arrowsmith*, personal communications), the Thai and Austrian isolates being clearly different from the others according to both electrofocusing and complement-fixation tests. It seems possible, therefore, that electrofocusing could be a useful aid for subtyping aphthoviruses. However, the technique does have limitations and these are discussed below.

Most of the mutations that can be detected by electrofocusing would not be expected to affect antigenic regions of the virus, and the extreme sensitivity of the technique to possibly trivial differences must be a disadvantage. The reverse problem of unrelated polypeptides having the same isoelectric point can also arise by chance. Thus both similarities and differences can give false clues to evolutionary relationships. The same problems apply to other methods of biochemical identification, especially to those involving one-dimensional analysis. A possible advantage of electrofocusing over RNA fingerprinting is that the isoelectric point does seem to be under the direct control of natural selection (Sect. 5). In practice, however, we know of no instance in which an isoelectric point can be used as a definitive diagnostic trait. We saw in Sect. 6 that it would be meaningless to classify aphthoviruses on the basis of the isoelectric point of even the most conserved capsid polypeptide, VP4. Early hopes of a type 0-specific VP2 proved equally false. Clearly a simple, descriptive approach to classifying aphthoviruses does not work.

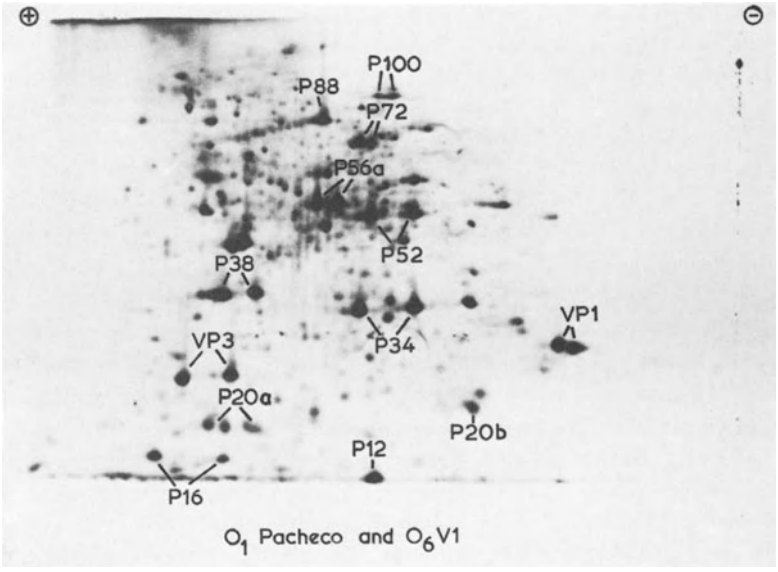

Fig. 10. A comparison of aphthoviruses by two-dimensional electrophoresis. Polypeptide extracts of the two strains were mixed and run as in Fig. 1

A more quantitative approach might be based on estimates of the genetic distance between viruses, calculated from the number of shared polypeptides (*Nei* and *Chakraborty* 1973). However, one-dimensional patterns give only a crude measure of genetic distance, since they contain only six independently coded polypeptides (P38 being the precursor of VP2 and VP4, and P72 being the precursor of P56a). By resorting to two-dimensional gels the number of polypeptides, and hence the statistical accuracy, is increased. Fig. 10 shows a comparison between the polypeptides of two different subtype strains of aphthovirus. Three independently coded polypeptides, P16, P12 and P20b, are revealed in addition to those visible on one-dimensional gels. In this example the two strains differ in P16, whereas P12 and P20b are conserved. It should be noted that the differences in Fig. 10 all lie in the horizontal (electrofocusing) dimension, the two subtypes being virtually indistinguishable by SDS gel electrophoresis.

The more genetic markers (e.g., polypeptides, RNA genome segments, T_1 oligonucleotides, DNA restriction fragments) that can be compared, the more accurate will be the estimate of genetic distance. For this reason we should expect RNA fingerprinting to be better than electrofocusing for classifying picornaviruses, and that electrofocusing would be more valuable for classifying large viruses that induce many different proteins than it is for picornaviruses.

8 Peptide Fingerprinting

Proteolytic treatment of SDS-denatured proteins produces peptides with molecular weights of 5000–30000 (*Cleveland* et al. 1977) which are amenable to electrofocusing (*King* et al. 1980). Provided the conditions of proteolysis are controlled, reproducible fingerprints can be obtained. Fig. 11 shows fingerprints of the coat proteins of several aphthovirus mutants. Most charge-change mutations show up as shifts in one or more of the peptides. We deduce from these fingerprints that the protease generates one peptide from VP1, which we call VP1α; two independent (i.e., non-overlapping) peptides from VP2, called VP2α and VP2β; and two independent peptides from VP3, called VP3α and VP3β. Different sets of fragments can be generated by using different proteases. Electrofocusing of cyanogen bromide cleavage fragments has also been reported (*Bibring* and *Baxandall* 1978). The peptides in Fig. 11 were visualized by staining, but they can also be radiolabelled (preferably in a common residue like leucine), and the technique is therefore applicable to non-structural polypeptides that have been eluted from polyacrylamide gels.

Peptide fingerprinting can be used in connection with any of the applications of electrofocusing described in this review. For example, by distinguishing between mutations that produce similar polypeptide shifts we were able to obtain biochemical evidence of genetic recombination between mutations belonging to the same aphthovirus strain (*King* et al. 1982a). Fingerprinting can also be used to check for spurious resemblances between polypeptides (Sect. 7). Moreover, since fragmentation of polypeptides increases the number of independent markers, it should be possible to determine evolutionary relationships more precisely by making use of comparative fingerprint data.

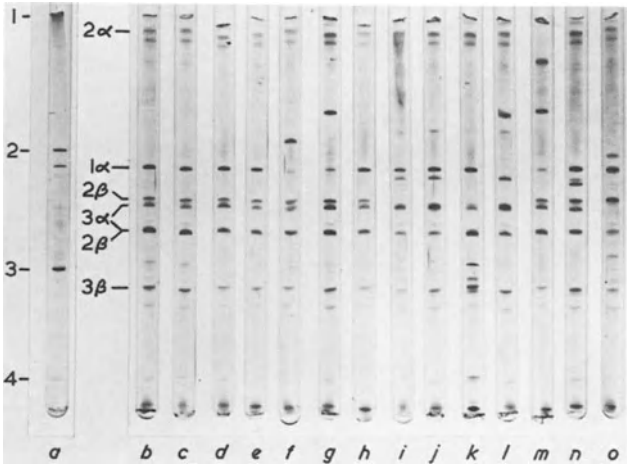

Fig. 11. Peptide fingerprints of the structural polypeptides of aphthovirus mutants. Polypeptides were disrupted in SDS and electrofocused as in Fig. 6. (*a*), ts^+; no treatment; (*b–o*), polypeptides digested with *Staphylococcus aureus* V8 protease; (*b*), ts^+, (*c*), ts^+, virus previously treated with trypsin to cleave VP1 to VP1L; (*d–g*), various ts mutants altered in VP1; (*h–k*), mutants altered in VP2; (*l, m*), mutants altered in both VP1 and VP2; and (*n–o*), mutants altered in VP3. (*King* et al. 1980)

9 Conclusions

Electrofocusing is a sensitive method for studying genetic change in viruses and has a wide range of applications. In general, similarities between virus strains have greater diagnostic significance than differences, because conservation of isoelectric point demands almost total sequence homology. Electrofocusing is therefore particularly effective as an epidemiological technique, especially for simple viruses such as aphthovirus where large numbers of isolates can be compared quickly and easily.

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A Sensitive Assay for Detection of Deoxythymidine Kinase and its Application to Herpesvirus Diagnosis

J. SIMON GRONOWITZ* AND CLAES F.R. KÄLLANDER*

1 Introduction

The herpesviruses comprise a group of very complex viruses. A number of antigens are encoded by the viral genome, some of which carry enzymatic functions. A method for measuring antibodies against a single antigen without previous protein purification is to determine the capacity of sera to block a specific enzymatic function. The human herpesviruses are known to encode their own DNA polymerases and deoxyribonucleases. Herpes simplex virus (HSV) and varicella zoster virus (VZV) have, in addition, their own deoxythymidine kinases (dTk) (for review see *Cheng* et al. 1979). The virus-encoded enzymes are immunogenic, and antisera prepared by immunization of animals with extracts from infected or transformed cells are capable of neutralizing the enzymatic activity. So far only a few studies have been reported concerning the occurrence of antibodies against virus-induced enzymes in connection with human infections. *Rawls* et al. (1974) screened a large number of human sera and found that they contained antibodies against HSV dTk only in exceptional cases. In contrast, *Cheng* et al. (1978) have reported that human sera frequently are positive for anti-

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bodies against HSV dTk. Antibodies against HSV and Epstein-Barr virus deoxyribonucleases have also been reported (*Cheng* et al. 1978, 1980).

We have designed a sensitive assay for the detection of dTk and antibodies against dTk. In this communication we summarize our studies using the optimized dTk assay for identification of herpesviruses in specimens and for detection of antibodies against herpesviruses. We show that the dTk assay can be used for rapid identification and typing of herpesviruses in clinical isolates. We moreover report that certain types of viral infections are connected with increased levels of serum dTk.

2 Materials and Methods

2.1 Clinical Specimens

Most sera and specimens were obtained from the virus diagnostic laboratory in the Department of Medical Virology, Uppsala University. Some sera have been provided by the National Bacteriological Laboratory in Stockholm as well as from physicians from different parts of Sweden. Complement-fixing (cf) antibody titers of sera were determined by standard procedures.

2.2 Enzyme Preparations

Detailed methods for preparation of dTk have been described before (*Gronowitz* and *Källander* 1980). Vesicle fluid specimens used for identification and typing were collected from patients with clinical HSV infections by rupturing the blisters with a needle and absorbing the secretion into a calcium alginate swab. The secretion was then eluted into 200 µl buffer and frozen at −20 °C before transport to the laboratory for analysis.

2.3 dTk and dTk-Inhibition Assays

Details of the dTk and dTk-inhibition assay procedures have been described elsewhere (*Gronowitz* and *Källander* 1980; *Gronowitz* et al. 1982). The dTk activity is determined by using [^{125}I]IUdR (specific activity, 200 Ci/mmol) as the substrate at a final concentration of 1×10^{-7} *M*. The amount of incorporated radioactivity is measured by pipetting duplicate samples onto Whatman DEAE papers, kept at 90 °C–100 °C.

In order to calculate dTk antibody titers, the residual dTk activities obtained after incubation of a given amount of enzyme with different serum dilutions were calculated and expressed as a percentage of the normal control (enzyme incubated with a negative serum or without serum) after correction for background. Sera giving less than 30% inhibition in a 1:1 dilution were considered to be negative. The titer of a positive serum is defined as the $\log_2$ dilution giving 30% enzyme inhibition.

2.4 Typing of Isolates by a dTk-Inhibition Assay

Cell extracts or blister secretions were diluted twofold and 5 µl was transferred from each dilution to three wells in a microtiter plate in order to obtain three identical

samples. Prediluted HSV type 1 dTk antibody-positive serum (rabbit origin; 25-μl aliquot) was added to the first well, a human HSV type 2 dTk antibody-positive serum to the second well, and a serum negative for dTk-neutralizing antibody to the third well. Residual enzyme activities were determined after 90 min incubation.

2.5 Use of dTTP for Inhibition of Cellular dTk

When determining viral dTk antibody titers, it is of importance to minimize the contribution of cellular dTk activity. For this reason 2×10^{-5} *M* deoxythymidine triphosphate (dTTP) was used in VZV dTk antibody assays and in all typing procedures. This concentration of dTTP blocks the cellular cytosolic dTk activity efficiently but has a negligible effect on herpesvirus dTk (*Källander* et al. 1982).

3 Results and Discussion

3.1 An Optimized Assay for Detection of dTk and Antibodies Against dTk

In order to design a highly sensitive assay for detection of dTk it is important to use a substrate which has a high turnover rate and which can be measured efficiently. We have previously shown that [^{125}I]IUdR can be used as a substrate by both HSV type 1 and type 2 dTk. The advantage of this radiolabeled substrate is that it can be obtained

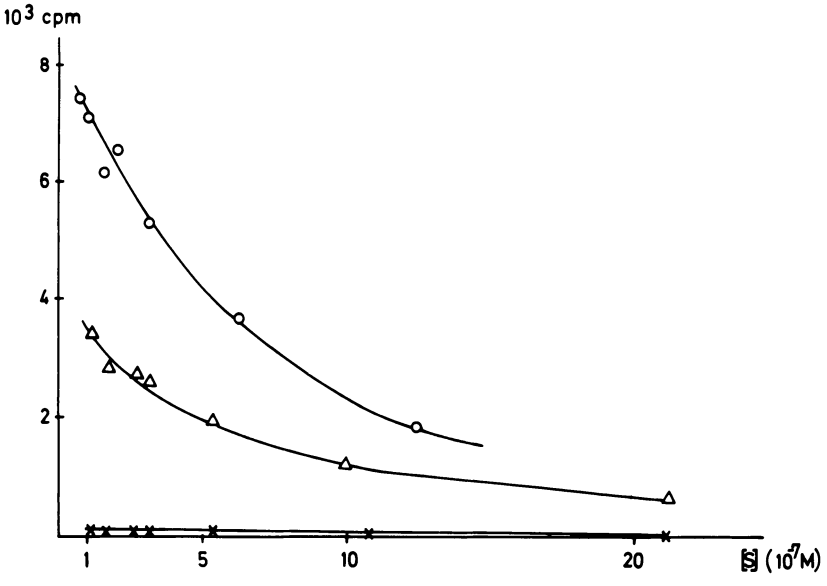

Fig. 1. Correlation between substrate concentration [*S*] and incorporation in the dTk assay, using an extract for HSV-1 (strain C42) as the source of enzyme. Each sample contained 0.32 μCi of isotope, and the values for incorporation of label refer to a 10-min assay period. (○——○), [^{125}IUdR] used as a substrate with enzyme from 10^2 HSV-infected cells; (x——x), [^{3}H]dT used as a substrate with enzyme from 10^2 HSV-infected cells; (△——△), [^{3}H]dT used as a substrate with enzyme from 1.3×10^3 cells. (*Gronowitz* and *Källander* 1980)

commercially with a very high specific activity and that the label can be measured with high efficiency. Moreover, viral dTk isozymes utilize IUdR more efficiently than thymidine, particularly at low substrate concentrations (*Gronowitz* and *Källander* 1980) (Fig. 1). The sensitivity of the assay can thus be increased by decreasing the substrate concentration. As compared with conventional assays which utilize [^{3}H]thymidine at a concentration of 10^{-5} *M* as a substrate, the optimized assay requires 450 times less enzyme for detection, and it can detect HSV dTk activity from as few as 1–10 infected cells.

Another important factor when assaying small amounts of enzyme in nonpurified samples is the selectivity of the assay for the viral enzyme. Due to its greater sensitivity to feedback inhibition by dTTP, the major cellular dTk-F isozyme can be inhibited by incorporating 2×10^{-5} *M* dTTP in the assay mixture, without significantly reducing the activity of the viral enzymes.

Recently the sensitivity of the assay has been increased further by using an alternative buffer system which stabilizes the cellular dTk-F and is beneficial for the viral dTk isozymes (*Gronowitz* et al., to be published). By use of this modified dTk assay it is possible to detect dTk in sera from normal individuals; furthermore, the assay is linear for at least 2 h when applied to detection of dTk activity in serum.

3.2 Antibodies Against HSV dTk in Human Sera

Samples of 100 human sera delivered to the virus diagnostic laboratory at Uppsala University were examined in order to study the distribution of antibodies against HSV dTk (*Gronowitz* and *Källander* 1981). For comparison of titers against HSV were also measured. The sera originated from 74 patients, only 7 of which were suspected of having an acute HSV infection. The results showed that 41 sera were HSV cf positive, whereas the remaining were negative. All sera were titrated against both type 1 and type 2 HSV dTk. The results showed that none of the cf-negative sera contained antibodies against either type 1 or type 2 HSV dTk. Sera which contained cf antibodies against HSV were found to consist of two groups, one positive and the other negative in the dTk-inhibition assay. Sera in the latter group were in nearly all cases found to be from patients who had experienced a recent primary infection. Among the sera which were positive for antibodies against HSV dTk, those which were collected from older individuals usually had higher titers.

The results described above indicate that patients who had experienced a recent primary HSV infection were negative for antibodies against dTk. Therefore it was decided to examine whether seroconversion could be observed under these conditions. The results showed that out of 19 patients 12 became dTk antibody positive 16–287 (average 179) days after the primary infection and moreover, that the dTk response exhibited good type-specificity. Recurrent infections did not appear to be needed in order to give a dTk antibody response, since only 2 of the 12 patients who became positive had experienced recurrences before seroconversion.

3.3 Immunological Specificity of the HSV dTk Antibody Response

The type-specificity of the antibody response against dTk was studied by titrating sera against both HSV type 1 and type 2 dTk. From the results it is apparent that different

levels of immunological cross-reactivity are observed for different sera (*Gronowitz* and *Källander* 1981). In most cases cross-reactivity is only apparent when the titer against one HSV dTk type is high. However, sera from some patients revealed approximately equal titers against both types of HSV dTk. The most likely interpretation of these results is that a weak cross-reactivity is apparent between HSV type 1 and type 2 dTk which becomes noticable when the antibody titer against one type of dTk is high. Patients having sera with equal titers against both types of dTk have probably experienced both types of HSV infections. It cannot, however, be ruled out that different subjects respond differently with regard to type-specificity.

3.4 Identification and Typing of HSV Infection by the Specificity of dTk Activity

Twenty HSV isolates, including one dTk-negative mutant strain, were coded blind before typing by the dTk-inhibition assay. The results obtained were in perfect agreement with those obtained using an independent method and also with the predictions which can be made from the clinical symptoms (Table 1). One isolate, the dTk-negative mutant, was impossible to type since it did not raise the dTk activity as compared with a preparation from mock-infected cells (Table 1).

In order to test whether the method could reveal dTk activity in vesicle fluid, specimens were collected from a few patients and analyzed in the presence or absence of dTk antibodies. The results, which were compared with those obtained by a conventional isolation procedure, showed that dTk could be detected and typed in seven of nine samples yielding virus by isolation (Table 2). Furthermore, dTk activity was detected in one sample which failed to give a positive isolation. Results from typing by the dTk-inhibition assay were always in complete agreement with results obtained in other assays and correlated with the clinical picture of the patient. It is thus apparent that the HSV dTk assay can be used for rapid detection and typing of clinical isolates.

3.5 Antibodies Against VZV dTk in Human Sera

Two categories of sera were examined for the presence of antibodies against VZV dTk:

1. Serum pairs from patients with clinical indications of varicella or herpes zoster showing a significant rise in VZV cf titer.

2. VZV cf-negative sera from ten varicella-immune and ten nonimmune patients. The results showed that antibodies against VZV dTk were present exclusively in sera from patients suffering from herpes zoster (Table 3). Neither acute or convalescence sera from varicella patients, nor cf-negative immune sera contained VZV dTk antibodies. Among the patients in the zoster group, one had VZV dTk antibodies in the cf-negative acute serum. Another zoster patient did not have dTk antibodies, which, however, may be related to the fact that this patient was under treatment with cytostatic drugs because of a malignant lymphoma.

Table 1. Typing of HSV isolates by the immunological specificity of their induced dTk. (*Gronowitz* et al. 1982)

Isolate	Enzyme activity control[a] (cpm × 10^{-3})	Residual enzyme activity after inhibition with serum (%)		Immunological type of dTk	Type by IUdR sensitivity	Type by CiEop[b]	Location of infection
		Type 1	Type 2				
C793	59	13.8	78.7	1	1	1	Mouth
C928	69	13.3	57.1	1	1	1	Lip
C156	12	9.5	79.0	1	1	1	Face
C777	18	15.9	108.9	1	1	1	Throat
C355	33	8.4	70.7	1	1	1	Throat
C356	55	6.4	96.7	1	1	1	Lip
C433	109	8.2	80.5	1	1	1	Mouth
C470	19	10.0	88.3	1	1	1	Throat
C420	49	5.1	73.8	1	1	1	Mouth
C131	66	4.8	68.0	1	Intermediate	1	Cheek
C884	54	67.7	26.7	2	Intermediate	2	Genital region
C700	43	64.4	37.0	2	Intermediate	2	Genital region
C915	1	168.3	199.4		2	1	Eye
D65	18	106.8	24.7	2	2	2	Genital region
C249	228	112.0	22.5	2	2	2	Genital region
C836	52	46.9	30.3	2	2	2	Genital region
C825	17	57.2	37.0	2	2	2	Hip
D218	50	57.2	30.2	2	2	2	Genital region
C43	14	103.3	46.0	2	2	2	Buttock
C411	255	81.1	38.3	2	2	2	Thigh
Cell[c]	1	268.0	244.0				

[a] Counts per minute per 30-min assay with 5 μl cell extract diluted 1:16 after incubation with HSV-negative serum. The specific activity of the IUdR was 100 Ci/mmol; [b] Counterimmunoelectro-osmophoretic (CiEop) method. (*Jeansson* 1972); [c] An enzyme preparation from unifected cells was used

Table 2. Detection and typing of HSV dTk activity in vesicle fluid. (*Gronowitz* et al. 1982)

Sample code	Enzyme activity[a] (cpm × 10^{-3})	Residual enzyme activity after inhibition with serum (%)		Immunological type of dTk	Virus isolation (days)[b]	Type according to isolate dTk	Location of blisters
		Type 1	Type 2				
1085	58	6.6	92.3	1	2	1	Face
1099							Genital region
1016	76	49.0	25.2	2	2	2	Genital region
1035	61	9.3	108.0	1	2	1	Face
1168	88	70.0	28.4	2	2	2	Genital region
1309					3	1	Face
1306					2	2	Genital region
1580	236	53.5	25.3	2	2	2	Genital region
1450	7	68.6	0	2			Genital region
1522(1)	8	15.9	118.4	1	4–5	1	Face
1521							Genital region
1522(2)							Face
1533	47	3.2	93.0	1	2	1	Face
621							Genital region
1613							Genital region

[a] Activity of control (secretion incubated with HSV-negative serum) using 5 μl undiluted secretion sample and IUdR at a specific activity of 370 Ci/mmol;
[b] Time elapsed before more than 70% of cells in the culture showed cytopathogenic effect

Table 3. dTk antibodies in serum specimens from patients with varicella- or herpes zoster and in serum specimens from VZV-immune and nonimmune healthy individuals. (*Källander* et al. 1982)

Group	cf VZV[b]	cf HSV[c]	VZV dTk[d] (log_2)
Typical varicella (8 pairs)	< 4	64	< 1.0[a]
	64	128	< 1.0
	< 4	< 4	< 1.0
	16	< 4	< 1.0
	< 4	< 4	< 1.0
	32	< 4	< 1.0
	< 4	< 4	< 1.0
	32	< 4	< 1.0
	< 4	< 4	< 1.0
	32	< 4	< 1.0
	< 4	16	< 1.0
	64	64	< 1.0
	< 4	8	< 1.0
	32	32	< 1.0
	< 4	8	< 1.0
	64	64	< 1.0
Typical herpes zoster (10 pairs)	16–32	16	< 1.0
	64	16	3.5
	4	16	< 1.0
	64	16	5.5
	4	4	< 1.0
	8	4	2.5
	4	< 4	< 1.0
	64	< 4	7.5
	8	16	< 1.0
	128	64	3.5
	4	4	< 1.0
	64	8	< 1.0
	4	16	3.5
	64	16	9.0
	4	8	< 1.0
	64	8	9.5
	4	32	< 1.0
	32	32	4.5
	16	8	7.5
	64	8	10.0
Immune (10 single sera)	4	ND	< 1.0
Nonimmune (10 single sera)	4	ND	< 1.0

[a] Titer <1.0 is considered to be negative; [b] Complement fixation titers to varizella zoster virus; [c] Complement fixation titers to herpes simplex virus; [d] Deoxythymidine kinase antibody titers to varizella zoster virus

3.6 Detection of Elevated Serum dTk Levels in Patients Suffering from Different Viral Diseases

While performing antibody inhibition assays with sera from different categories of patients, it was noticed that the serum in a few cases contained dTk activity. After examining the medical records from these patients, it became apparent that there was a correlation between serum dTk and clinical symptoms of infectious mononucleosis. In order to establish this correlation more firmly we examined several serum pairs collected from patients suspected of suffering from infectious mononucleosis. The results showed that sera collected during the acute stage of infectious mononucleosis always contained elevated levels of serum dTk (Table 4), in many cases 20–50 times the normal value. By analysis of serum pairs it was furthermore shown that the levels were normalized 4–6 weeks after the first symptoms were noticed.

Analysis of serum pairs from patients suffering from other types of viral disease revealed that elevated levels of serum dTk were commonly present in sera of patients suffering from rubella, morbilli, varicella, cytomegalovirus, and occasionally herpes simplex virus infection.

In order to characterize the serum dTk activity, an electrophoretic study was carried out. Selected sera containing elevated levels were studied and the positions of the peaks were correlated to those of the known cellular dTk isozymes. The results showed that the serum activity, except in sera from varicella patients, migrated with $R_f = 0.18$, which is the position of the cellular dTk-F activity.

Another way to discriminate viral dTk from the major cellular dTk activity is to use cytidine triphosphate (CTP) as the phosphate donor, since only the viral isozymes can utilize this phosphate donor. This discriminating assay was applied to the dTk activities which were present in serum, and the results showed, except for the varicella

Table 4. dTk activities in serum pairs from patients with infectious mononucleosis[a]

Age (Years)	Time after onset of illness (Days)	dTk activity (Units)	Paul Bunnell (Titer)
20	2	82	640
	60	6	40
16	5	98	80
	25	11	160
20	6	75	160
	53	7	80
42	13	63	320
	30	7	160
12	12	76	40[b]
	37	4	40
40	4	38	20[b]
	64	4	20
15	5	28	20[b]
	35	9	20

[a] The normal value is 2.4 units; [b] The diagnosis was verified by the fluorescent antibody technique

patients, that the serum dTks from other categories of patients were unable to use CTP as a phosphate donor. Taken together the results strongly indicated that the serum dTk activity which is present in connection with infectious mononucleosis and other viral infections is caused primarily by cellular enzymes. The most likely interpretation of the results is that the viral diseases which give rise to elevated serum dTk levels are accompanied by a lymphoproliferative response; the enzyme is thus present because of cell proliferation in combination with cell damage and cell death. More recent results also show that serum dTk is a valuable marker to study the progression of several types of human tumor diseases (*Gronowitz* et al., to be published).

4 Conclusions

We have designed a sensitive assay for detection of dTk in cells infected with HSV and VZV. The assay is capable of detecting dTk activity from 1–10 HSV-infected cells and can also measure dTk activity in human serum.

The assay has been used to screen human sera for antibodies against different herpesviruses, and the presence of type-specific antibodies against HSV type 1, HSV type 2, and VZV dTk isozymes was demonstrated. In the case of HSV infections it was furthermore shown that the occurrence of antibodies against dTk is considerably delayed in relation to the appearance of cf antibodies. The absence of dTk antibodies in sera from HSV cf-positive individuals is therefore related to a recent primary infection. Antibodies against the VZV dTk were found exclusively in sera from patients suffering from herpes zoster.

The type-specific nature of antibodies against the dTk isozymes also makes it possible to use the dTk-inhibition assay for typing of isolates. The method has been used for direct analysis of blister secretions without previous virus isolation, and the results of typing can be obtained within 5 h.

An analysis of dTk activity in human sera revealed elevated serum dTk activity during the acute stages of infectious mononucleosis which disappears 4–6 weeks after the onset of the illness. Moderately enhanced levels of serum dTk were also found in connection with morbilli, rubella, varicella, cytomegalovirus, and HSV types 1 and 2 infections. The potential use of the dTk assay for diagnostic purposes is discussed.

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The Characterization of Influenza A Viruses by Carbohydrate Analysis

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1 Introduction

The hemagglutinin and the neuraminidase of influenza viruses are glycoproteins of great importance for the biology of the virus and the nature of the disease. They play an essential role in the initiation of infection. It is generally agreed that the hemagglutinin is responsible for adsorption of the virus to the cell surface. In addition, the hemagglutinin is involved in penetration by triggering fusion of the viral envelope with cellular membranes. There is evidence that the neuraminidase also takes part in the latter reaction (*Huang* et al. 1980a, b, 1981). Both glycoproteins are also the surface antigens of the virus and due to their structural variability, they appear to be directly responsible for the uncontrolled recurrence of influenza epidemics in man. It is therefore not surprising that the structure of the hemagglutinin, as the major surface antigen, has been analyzed in detail. The complete amino acid sequences of a whole series of influenza A virus hemagglutinins have been determined (for review see *Ward* 1981), and the tertiary structure of the molecule has also been elucidated (*Wilson* et al. 1981).

Glycosylation of the influenza virus glycoproteins is initiated by the en bloc transfer of mannose-rich oligosaccharides from dolichol pyrophosphate to the polypeptide, where they are attached by *N*-glycosidic linkages to asparagine residues (*Schwarz* et al. 1977; *Nakamura* and *Compans* 1979a; *Keil* et al. 1979), as is the case with other viral glycoproteins (*Sefton* 1977; *Li* et al. 1978; *Robbins* 1979). Subsequently, some of the side chains undergo extensive modifications involving the removal of mannose and attachment of galactose and fucose residues. Thus, two major types of carbohydrate side chains are found on the glycoproteins: the mannose-rich type II containing only mannose and glucosamine, and the complex type I containing in addition galactose

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and fucose (*Schwarz* et al. 1977; *Nakamura* and *Compans* 1978; *Collins* and *Knight* 1978). Both types appear to be heterogeneous. This is suggested by our observations that subtypes, designated Ia and Ib and IIa and IIb, can be distinguished when glycopeptides obtained after pronase digestion of the glycoproteins are analyzed by gel filtration (*Keil* et al. 1979). Evidence for heterogeneity of types I and II side chains has also been obtained when hemagglutinin fragments prepared by cyanogen bromide cleavage (*Ward* and *Dopheide* 1980) and trypsin digestion (*Nakamura* et al. 1980) were analyzed. A so-called intermediate oligosaccharide type that consists of glucosamine, mannose, and galactose and is resistant to endoglycosidase H has been found on the hemagglutinin of the WSN strain (*Nakamura* et al. 1980).

In this contribution we will briefly describe the topographical analysis of the carbohydrate side chains on the fowl plague virus (FPV) hemagglutinin H7. We will then compare the oligosaccharide patterns of 21 influenza A strains of mammalian and avian origin containing all serotypes of hemagglutinin and neuraminidase known to date.

2 Structure and Location of the Carbohydrate Side Chains on the Fowl Plague Virus Hemagglutinin

Based on data from glycopeptide analyses and from glycosylation-inhibition studies, we had previously estimated that the FPV hemagglutinin contains five to six oligosaccharide side chains and that two of these are located on the HA_2 subunit (*Schwarz* et al. 1977). The elucidation of the amino acid sequence revealed that the polypeptide has a total of seven potential carbohydrate attachment sites (*Porter* et al. 1979). In order to localize the side chains we followed a procedure described schematically in Fig. 1. Fragments of the hemagglutinin were prepared by treatment with cyanogen bromide, V8 protease from *Staphylococcus aureus*, and trypsin. The position of the fragments was determined by radioactive labeling of the sugars and of specific amino acids. For instance, two cyanogen bromide fragments containing carbohydrate attachment sites were obtained from HA_2. One of these can be labeled in both tryptophan and tyrosine, whereas the other one contains only tyrosine. The results of this study are summarized in Fig. 2. The HA_1 subunit has four side chains which are all of type I; one potential attachment site (asparagine 231) is not glycosylated. Both attachment sites of HA_2 are glycosylated; the only type II side chain of the hemagglutinin is attached to asparagine 406, whereas asparagine 478 has a type I side chain.

The structure of the type II side chain has been analyzed in some detail. This chain exhibits considerable heterogeneity as demonstrated by the observation that four different high-mannose (HM) oligosaccharides (HM 1–4) can be discriminated after digestion with endoglycosidase H (Fig. 3). Analysis of the permethylated oligosaccharides in conjunction with studies employing endoglycosidase D has revealed that HM2, HM3, and HM4 possess 7, 8, and 9 mannose residues, respectively, and the common core structure shown in Fig. 4.

Our data indicate that at a given glycosylation site, only one type of side chain is present. Thus, the type II side chain of the FPV hemagglutinin is always attached to asparagine 406. Type I side chains are never observed in this position. There is evidence that the side chains of type I located in different positions on the polypeptide

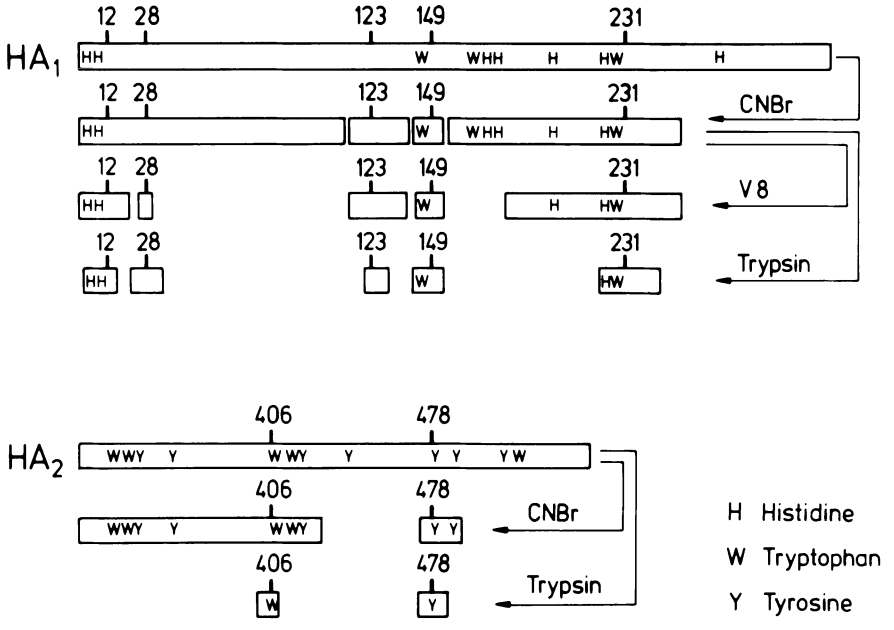

Fig. 1. Fragmentation of the FPV hemagglutinin used for identification of the carbohydrate side chains. Cyanogen bromide (*CNBr*) fragments were isolated by polyacrylamide gel electrophoresis. Tryptic fragments were isolated by chromatography on Biogel P6 and AG 50 × 2 columns. *Staphylococcus aureus* V8 protease digests were analyzed by polyacrylamide gel electrophoresis. The positions of all potential attachment sites and of some of the amino acids used in radioactive labeling are indicated. (From *Klenk* et al. 1981)

are not identical, as indicated by differences in sugar composition and sulfation (*Ward* and *Dopheide* 1980; *Nakamura* et al. 1980; *W. Keil*, manuscript in preparation). Thus, each glycosylation site has a specific carbohydrate side chain. However, Fig. 4 demonstrates that a specific side chain may exhibit considerable microheterogeneity. Structural analysis of the hemagglutinin oligosaccharides is therefore a rather complicated project.

3 Strain-Specific Variations in the Glycosylation Pattern

Although the complete amino acid sequence of the hemagglutinin and thus the positions of the potential carbohydrate attachment sites have been elucidated with a considerable number of influenza A strains, there are only few hemagglutinins for which information is available on the nature of the carbohydrate attached to a specific glycosylation site. Besides the FPV hemagglutinin, these are the hemagglutinins of strains A/Japan/305/57 (H2) and A/Memphis/102/72 (H3). Fig. 5 demonstrates that some attachment sites appear to be conserved (e.g., those around sequence positions 40, 50, and 510), whereas others show a high degree of variability. It is interesting to note that in the three-dimensional model of the hemagglutinin (*Wilson* et al. 1981), the conserved side chains, even though on opposite ends of the unfolded polypeptide, are located in proximity to each other at the base of the spike. This may indicate that

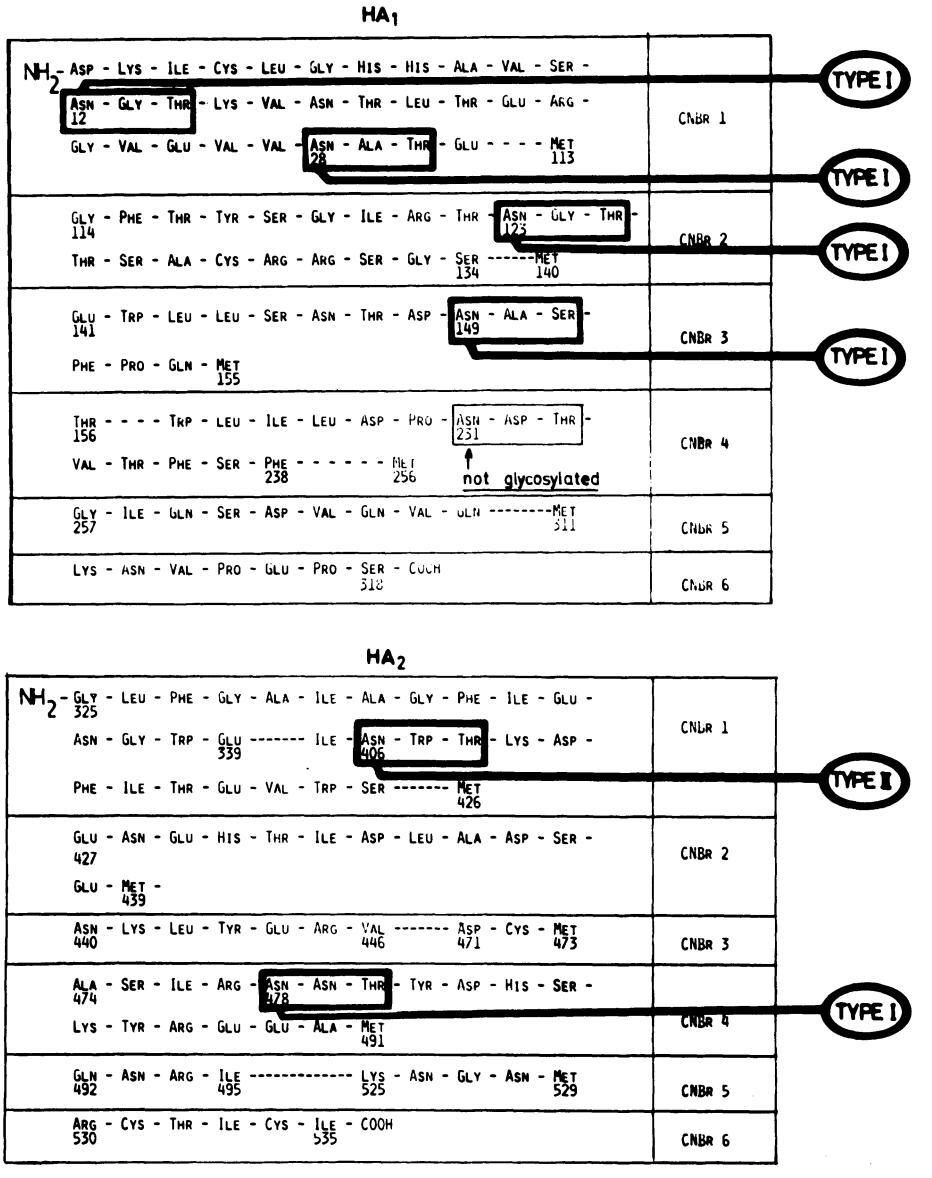

Fig. 2. Positions of the oligosaccharides on the hemagglutinin of strain A/FPV/Rostock/34. The amino acid sequences (*Porter* et al. 1979) at the N-terminal and C-terminal ends of the cyanogen bromide (*CNBr*) fragments and at the glycosylation sites are indicated. (From *Klenk* et al. 1981)

these side chains have a special function in maintaining the structure of the hemagglutinin.

A widely used procedure for analysis of the carbohydrate moiety of a glycoprotein involves digestion of the polypeptide by pronase followed by gel chromatographic analysis of the oligosaccharide side chains still linked to a small peptide fragment. This approach does not allow the precise localization of the individual side chains on the hemagglutinin, as does the procedure described above, but it is simple and still

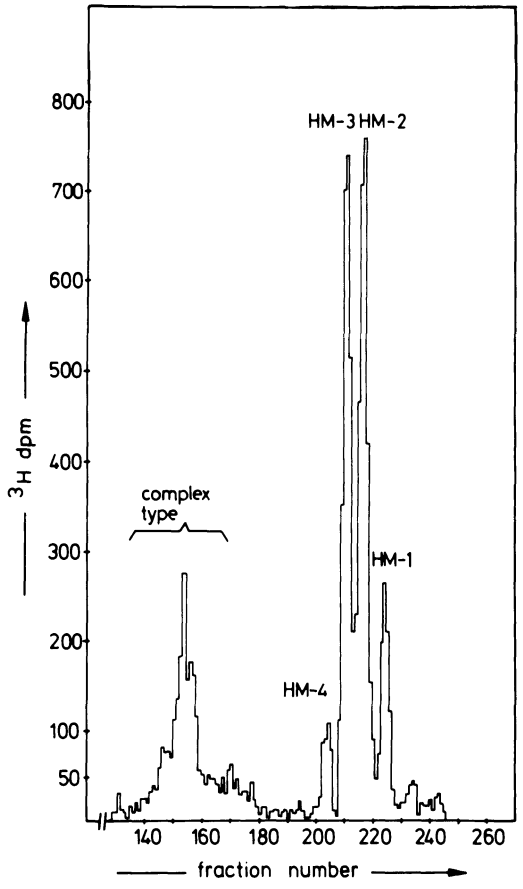

Fig. 3. Type II oligosaccharides of the FPV hemagglutinin. Pronase digests of HA_2 were subjected to endoglycosidase H treatment. The oligosaccharides derived from the type II side chain (*HM-1, 2, 3, 4*) were separated from type I glycopeptide (not susceptible to endoglycosidase H) by chromatography on a Biogel P4 column

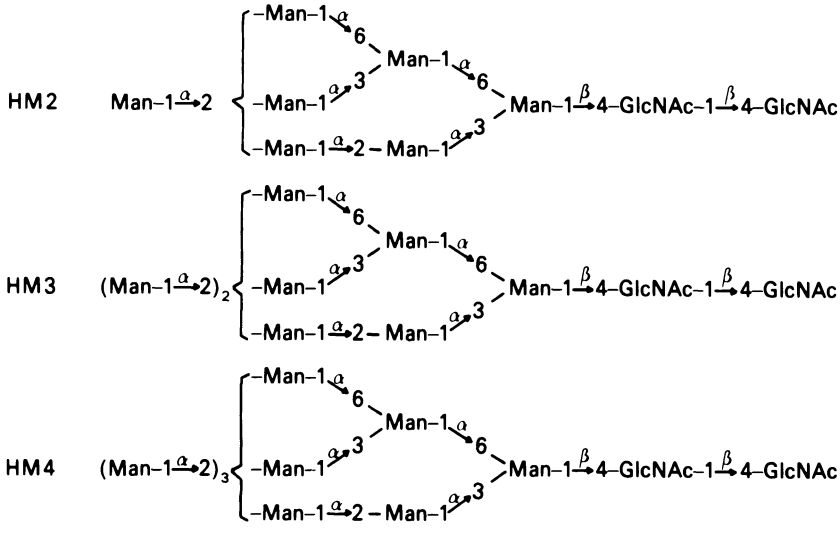

Fig. 4. Structural heterogeneity of the oligosaccharide attached to asparagine 406 of the FPV hemagglutinin. Oligosaccharides HM2, HM3, and HM4 (Fig. 3) were permethylated and hydrolyzed. The constituent sugars were analyzed by gas-liquid chromatography combined with mass spectrometry. *Man*, mannose; *GlcNAc*, *N*-acetylglucosamine

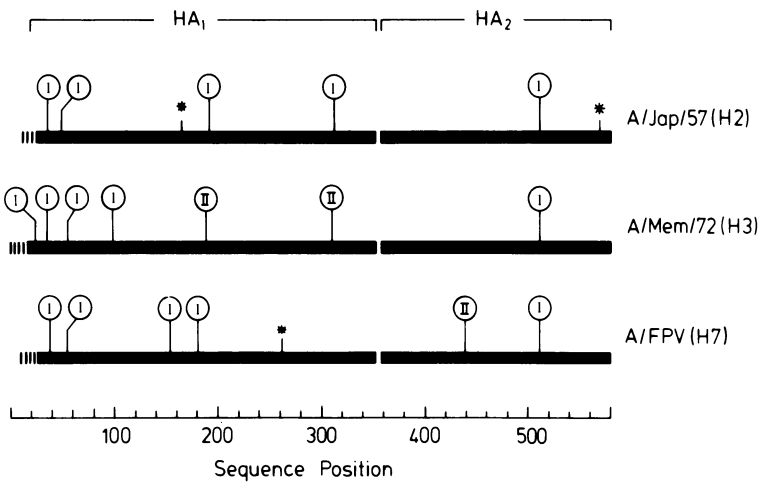

Fig. 5. Arrangement of carbohydrate side chains on three different hemagglutinins. The data on the H2 hemagglutinin are from *Waterfield* et al. (1980), *Klenk* et al. (1980), and *Ward* and *Dopheide* (1981). The data on the H3 hemagglutinin are from *Ward* and *Dopheide* (1980). Cysteine residues have been used for the alignment of the polypeptide chains. *I* and *II* designate side-chain type. *, not glycosylated

gives valuable information on strain-specific variations. With most strains we have observed type I and type II glycopeptides, each of which could be subdivided quite consistently into the subtypes Ia and Ib, and IIa and IIb, respectively (*Keil* et al. 1979). The proportions of the glycopeptides varied significantly with different strains. As an example, the glycopeptide patterns of the glycoproteins of strain A/chick/Germany/N49 (H10N7) and A/PR/8/34 (H1N1) are shown in Fig. 6. The HA_1 subunit of the H10 hemagglutinin contains predominantly type I side chains, whereas both types appear to be present in about equal amounts on the HA_2 subunit of this hemagglutinin. In contrast, both subunits of the H1 hemagglutinin contain only type I side chains. The neuraminidases of A/PR/8/34 and A/chick/Germany/N/49 contain both type I and type II glycopeptides. This was a consistent observation with all neuraminidase subtypes analyzed.

The results of the glycopeptide analysis of different hemagglutinins are summarized in Table 1. This list includes most serotypes known to date. The data demonstrate that on HA_1, which contains most of the carbohydrate side chains of the hemagglutinin (Fig. 5), type I oligosaccharides are prevalent. An exception is the H3 hemagglutinins, which all contain a substantial amount of type II in addition to type I side chains. This observation agrees well with the chemical analysis of other hemagglutinins of this serotype, in which two of the five or six side chains of HA_1 were found to be of the mannose-rich type (*Ward* and *Dopheide* 1981; see also Fig. 5). Thus it appears that the presence of a relatively high proportion of type II oligosaccharides on the HA_1 subunit is a general feature of the H3 serotype, regardless of whether the virus is of human, equine, or avian origin. The available evidence indicates that with a few exceptions the HA_2 subunit contains a single side chain (*Schwarz* and *Klenk* 1981) which is of the complex type (Table 1) and highly conserved, as already pointed out above. The exceptions are H8, in which HA_2 appears to be completely carbohydrate-

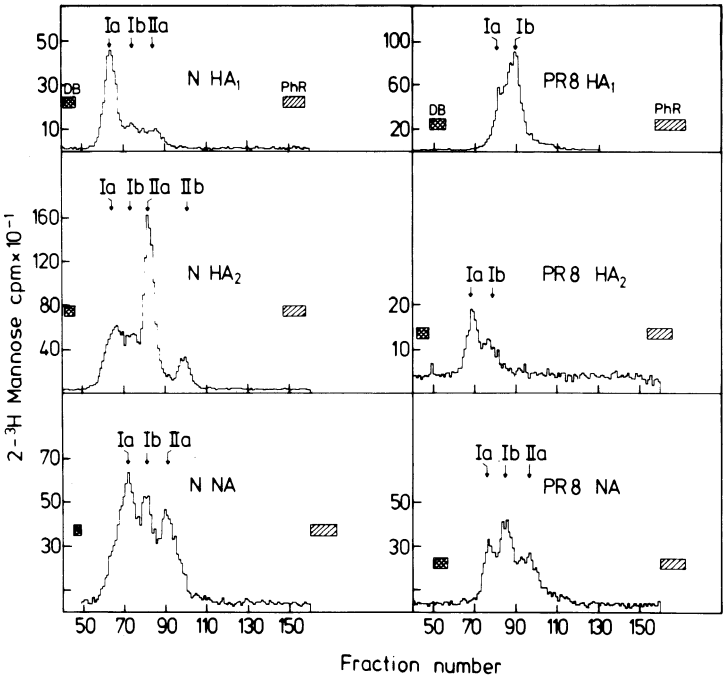

Fig. 6. Glycopeptides derived from the glycoproteins of strains A/chick/Germany/N/49 (H10N7) and A/PR/8/34 (H1N1). Glycoproteins were isolated from purified virus labeled with [2-^{3}H]mannose. Glycopeptides were prepared by pronase digestion of the glycoproteins and analyzed on Biogel P7 columns. Fractions in which dextran blue (*DB*) and phenol red (*PhR*) eluted are indicated. *NA*, neuraminidase. (From *Schwarz* and *Klenk* 1981)

free, and H5, in which the HA_2 glycopeptide is indistinguishable in size from a type I glycopeptide (data not shown) but free of fucose (Fig. 7). Thus, H5 may have an oligosaccharide side chain of the intermediate type linked to HA_2. The H7 and H10 hemagglutinins have an additional side chain on the HA_2 subunit which belongs to the mannose-rich type. It is interesting to note that this similarity in carbohydrate patterns reflects a close relationship in the amino acid sequences of both hemagglutinins (*Air* 1981).

The easiest approach to detect strain-specific variations in carbohydrate composition is the following one. Since the oligosaccharide side chains of HA_1 and HA_2 differ in both number and composition, the constituent sugars are distributed in specific proportions between both hemagglutinin fragments. This can be demonstrated by polyacrylamide gel electrophoretic analysis of virus labeled with radioactive sugars (Fig. 7). The distribution of the individual sugars can be calculated as the ratio of radioactivity incorporated into the HA_1 subunit to that incorporated into the HA_2 subunit. These ratios vary with different viruses, again indicating that strain-specific variations in the carbohydrate composition exist (Table 2). Variations in the ratios are most evident between hemagglutinins of different serotypes. However, occasionally even within the same serotype differences are observed, e.g., in the fucose ratios of the H7 hemagglutinins. That the sugar ratios are indicative of the actual oligosaccharide composition of HA_1 and HA_2 becomes quite clear if they are compared

Table 1. Glycopeptides derived from hemagglutinin subunits HA_1 and HA_2

Strain	Serotypes of hemagglutinin and neuraminidase	Glycopeptides[a] from			
		subunit HA_1		subunit HA_2	
		I	II	I	II
A/PR/8/34	H1N1	++	–	++	–
A/FM/1/47	H1N1	++	–	++	–
A/swine/Shope/31	H1N1	++	–	++	–
A/Singapore/1/57	H2N2	++	–	++	–
A/Port Chalmers/1/73	H3N2	++	++	++	–
A/equine/Miami/1/63	H3N8	++	++	++	–
A/duck/Ukraine/1/63	H3N8	++	++	++	–
A/duck/CSSR/56	H4N6	++	+	++	–
A/turkey/Ontario/7732/66	H5N9	++	+	++[b]	–
A/chick/Scotland/59	H5N1	++	+	++[b]	–
A/FPV/Rostock/34	H7N1	++	–	++	++
A/FPV/Dutch/27	H7N7	++	–	++	++
A/fowl/Victoria/75	H7N7	++	–	++	++
A/turkey/Oregon/71	H7N3	++	–	++	++
A/turkey/Ontario/6116/68	H8N4	++	+	–	–
A/turkey/Wisconsin/1/66	H9N2	++	+	++	–
A/chick/Germany/N/49	H10N7	++	+	++	++
A/duck/England/56	H11N6	++	–	++	–
A/duck/Alberta/60/76	H12N5	++	+	++	–

[a] I and II, type of side chain; [b] intermediate type; ++ major glycopeptide peak; + minor glycopeptide peak

with the glycopeptide patterns mentioned above. Thus, the presence of type II side chains in the HA_1 subunit of H3 hemagglutinins and in the HA_2 subunit of H7 and H10 hemagglutinins is clearly reflected by the respective mannose ratios.

4 Conclusions

The data presented here demonstrate wide variations in the oligosaccharide pattern of influenza A virus hemagglutinins. These variations were usually quite distinct between different serotypes. However, we have also found slight differences between strains of the same serotype, as has been observed before by *Nakamura* and *Compans* (1979b). This observation is not surprising in the light of recent sequence analyses of several hemagglutinins of the H3 serotype (*Ward* 1981) which demonstrate that

Table 2. Distribution of radioactive sugars between hemagglutinin subunits HA_1 and HA_2

Strain	Serotypes of hemagglutinin and neuraminidase	Ratio HA_1/HA_2			
		Man	GlcN	Gal	Fuc
A/PR/8/34	H1N1	4.6	3.7	3.0	5.1
A/FM/1/47	H1N1	3.4	3.3	3.2	3.4
A/swine/Shope/31	H1N1	5.0	5.1	3.6	2.4
A/Singapore/1/58	H2N2	4.1	3.7	2.2	7.7
A/Port Chalmers/1/73	H3N2	8.1	4.5	3.3	2.3
A/Victoria/3/75	H3N2	9.4	3.9	3.7	2.9
A/equine/Miami/1/63	H3N8	7.0	4.1	3.1	1.9
A/duck/Ukraine/1/63	H3N8	7.3	3.7	4.2	2.1
A/duck/CSSR/56	H4N6	4.9	2.8	2.6	2.3
A/turkey/Ontario/7732/66	H5N9	5.6	4.7	3.6	∞
A/chick/Scotland/59	H5N1	4.5	N.D.	2.3	∞
A/turkey/Canada/63	H6N8	3.0	2.0	2.3	4.6
A/FPV/Rostock/34	H7N1	1.2	3.0	2.4	3.5
A/FPV/Dutch/27	H7N7	1.0	2.5	2.4	4.0
A/fowl/Victoria/75	H7N7	1.1	2.1	2.4	1.6
A/turkey/Oregon/71	H7N3	1.1	2.4	2.7	2.5
A/turkey/Ontario/6116/68	H8N4	∞	∞	∞	∞
A/turkey/Wisconsin/1/66	H9N2	3.4	4.7	3.3	2.5
A/chick/Germany/N/49	H10N7	1.0	1.8	2.2	2.0
A/duck/England/56	H11N6	2.8	7.2	5.0	7.6
A/duck/Alberta/60/76	H12N5	3.6	3.3	2.6	2.0

Man, mannose; GlcN, glucosamine; Gal, galactose; Fuc, fucose

elimination or introduction of possible attachment sites as a result of single base exchanges is a frequent event in the course of antigenic drift. The diversity in the oligosaccharide patterns of the hemagglutinins of different influenza A strains all grown in the same host strengthens the concept that the primary structure of the polypeptide is an important determinant for the carbohydrate moiety of the glycoprotein.

In addition to serological and other biochemical techniques, the analysis of carbohydrate patterns may therefore become a standard procedure for the characterization and differentiation of influenza and other enveloped viruses.

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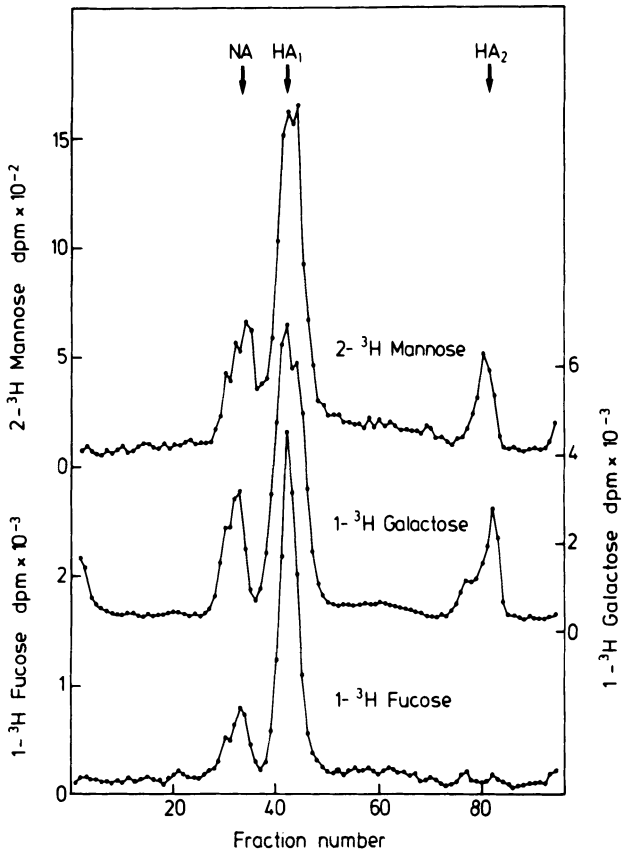

Fig. 7. Incorporation of radioactive sugars into the glycoproteins of strain A/turkey/Ontario/7732/66 (H5N9). Purified virus labeled with [2-^{3}H]mannose, [1-^{3}H]galactose, or [1-^{3}H]fucose was analyzed by polyacrylamide gel electrophoresis. *NA*, neuraminidase. (From *Schwarz* and *Klenk* 1981)

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The Application of RNA Fingerprinting and Sequencing to Viral Diagnosis

DAVID H.L. BISHOP*

1 Introduction

More than 600 different virus serotypes have been shown to infect animals. Fortunately only a minority are the etiologic agents of disease in man. Despite this, they are of major concern since currently only a few viral diseases can be cured and only a few can be prevented by vaccination.

Viruses change and evolve by a variety of means. These include recombination, point mutation, sequence repetition, deletion, or inversion. For RNA viruses with segmented genomes (arenaviruses, bunyaviruses, orthomyxoviruses, and reoviruses), reassortment of the segments of two compatible viruses can yield new recombinant viruses. Recent studies indicate that for foot-and-mouth disease, virus recombination can occur within an RNA segment (*A. King*, personal communication). Recombinant viruses may invade hosts that have not previously experienced infection by one or other parental virus and therefore lack antibody protection. Such a situation is believed to be a major factor in the periodic occurrence of new pandemics of influenza.

Point mutations and other sequence changes are basically caused by mistakes made by the replication enzymes of a virus. Misreading an RNA template is probably a useful (and inherent) attribute of an enzyme since it produces variant genotypes on which selection can draw. Virus variants can have a selective advantage over a parental virus (e.g., due to a change in an antigenic site allowing them to break through antibody repression). Variant viruses may also emerge not because of some inherent selective advantage but because of chance cloning in a particular host or vector that leads to more effective virus spread. Understanding how new viruses emerge and the causes of virus evolution is important not only for relating new isolates to antecedent viruses, but also for determining the factors that lead to the selection of new strains.

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Virus isolates are continually being obtained. On the basis of standard serological tests (hemagglutination inhibition, complement fixation, neutralization of infectivity), many of the new isolates can be categorized as subtypes or varieties of a known virus serotype since they exhibit an antigenic difference. Other virus isolates are frequently described as variants of a particular virus serotype (or its subtypes) since although they exhibit no demonstrable difference in standard serologic tests, in more exacting tests (e.g., the kinetics of antibody-mediated neutralization of infectivity), they can be shown to be slightly different. They are therefore described as serological variants. Still other isolates are antigenically indistinguishable by comparison with an antecedent virus. At the genome level, however, these antigenically identical isolates can often be distinguished from an earlier isolate because of a sequence change that does not cause a change in a measurable antigen. Such isolates are described as genotypic variants. Because of the evolution of viruses in nature, and the continual search for the etiologic agents of disease, it is inevitable that more virus isolates (genotypic and serological variants, new virus varieties, subtypes, and serotypes) will be obtained in the future.

The genomes of several segmented and nonsegmented RNA viruses have been characterized by fingerprinting. Particular agents that have been analyzed include selected arenaviruses, bunyaviruses, caliciviruses, paramyxoviruses, picornaviruses, oncornaviruses, orthomyxoviruses, reoviruses, rhabdoviruses, and togaviruses (*Clewley* and *Bishop* 1982). As discussed below, fingerprinting is primarily useful in the comparison of closely related viruses (i.e., those categorized as genotypic variants). It should be borne in mind in the following discussion that the serologic definitions given above are operationally defined and can have exceptions (e.g., a serological variant may exhibit only a few genomic changes and so resemble other isolates that are categorized as genotypic variants).

For only a few RNA viruses (oncornaviruses, picornaviruses, and orthomyxoviruses) has the complete nucleic acid sequence of a particular virus isolate been reported. Sequence information, although labor intensive, provides much more definitive data on virus relationships and evolution. Such data allow useful comparisons to be drawn not only between virus variants (genotypic and serological), varieties, and subtypes of a prototype strain, but also to a lesser degree between different serotypes of a viral serogroup, genus, or family. Sequence data also provide fundamental information on the coding, translation, transcription, and replication strategies of a virus.

Viral diagnosis has the goal of not only determining the identity of a virus associated with an infection but also its origins and relationship to other known agents. The purpose of this review is to show how fingerprinting and RNA sequencing can contribute to viral diagnosis and to the delineation of virus distribution and evolution in nature.

2 Fingerprinting the Genomes of RNA Viruses

The process of fingerprinting an RNA involves the preparation and purification of a radiolabeled RNA species, its digestion with a specific ribonuclease, and resolution of the resulting products on a two-dimensional support. The details and a discussion of several procedures that can be used to fingerprint RNA are given by *Clewley* and *Bishop* (1982).

Since a heteropolymeric RNA contains a specific sequence of ribonucleotides, digestion by an enzyme produces a specific set of RNA fragments (oligonucleotides). For ribonuclease T_1, which cuts only at guanylic acid residues, the derived oligonucleotides have the general formula $(A_{0-x}C_{0-y}U_{0-z})G_{0-1}$. Only the 3′ end sequence of an RNA can yield an oligonucleotide lacking a G residue. Small oligonucleotides (e.g., AG, AUG) are obtained from almost all RNA species; therefore they are not unique to a particular RNA. Large oligonucleotides (e.g., $U_{30}G$) are much more likely to be unique to an RNA species and together with other large oligonucleotides, characterize the ribonuclease T_1 digestion products. In general, the values for x, y, and z (for A, C, and U respectively), as well as the number of large oligonucleotides obtained from an RNA species, depend on the complexity of the RNA and the lengths of sequence bounded by G residues. For a heteropolymeric sequence of 10000 nucleotides having approximately equimolar amounts of all four bases (A, C, G, and U), it is common to obtain ribonuclease T_1-derived oligonucleotides that are up to 40 nucleotides in length. For such an RNA one usually obtains around 80 oligonucleotides that are greater than 13 nucleotides in length (*Clewley* and *Bishop* 1982), representing some 8%–12% of the RNA. Alternative preparations or an RNA species after ribonuclease T_1 digestion yield the same set of large oligonucleotides. However, preparations of other RNA species, even those of similar complexities, will yield a different set of large oligonucleotides. For RNA species containing a high proportion of G residues (e.g., ribosomal RNA) fewer large oligonucleotides are obtained (*Vezza* et al. 1978, 1980).

By resolving the derived oligonucleotides on a two-dimensional support using both their intrinsic charge (first dimension) and size (second dimension) as the prime factors in their resolution, the large oligonucleotides can be resolved into a characteristic pattern (fingerprint). Because each nucleotide (A, C, G, U) has a slightly different charge at pH 3.5, first dimension electrophoresis at this pH allows similar-sized oligonucleotides to be separated (e.g., $A_{15}G$ from $C_{15}G$, or $U_{15}G$). Also oligonucleotides which differ by only a single base substitution can be differentiated (e.g., $A_{14}UG$ from $A_{15}G$). Therefore, not only can completely different RNA species be distinguished on the basis of their fingerprints, but also variant sequences can be distinguished from parental species (provided they have at least one base substitution in a large unique oligonucleotide).

An example of the ability of fingerprinting to detect single nucleotide changes in an RNA sequence is provided in Fig. 1. The rhabdovirus, vesicular stomatitis virus (VSV, Indiana serotype) was grown in the presence of 5-fluorouracil (a mutagen), the progeny virus cloned, and the process repeated (*Clewley* and *Bishop* 1979). By comparison with the RNA fingerprint of the zero passage virus (Fig. 1A) the fingerprint of the virus obtained after nine consecutive mutageneses exhibited three changes, one new oligonucleotide (Fig. 1B) and two missing oligonucleotides (Fig. 1B). Since the new oligonucleotide was to the right of one of the missing oligonucleotides it probably represented a C to U or C to A point mutation. The other missing oligonucleotide may have involved an A (or C or U) nucleotide change to a G residue since there are apparently no other new large oligonucleotides, and the introduction of a G residue into a sequence would result in smaller (faster moving) oligonucleotides after ribonuclease T_1 digestion. In addition to these inherited changes, other changes in the fingerprint were evident by later passages (see Fig. 1C, D). The fingerprints of viruses

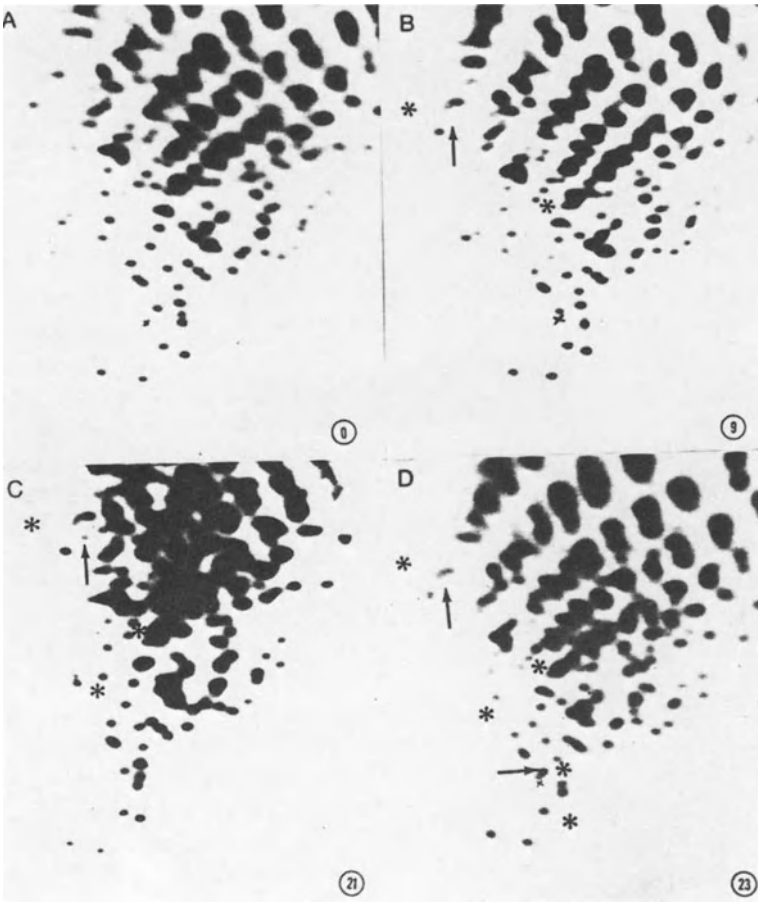

Fig. 1A–D. The effect of consecutive high-level mutagenesis on the genome oligonucleotide fingerprint of VSV Indiana. A) Starting clone; B) 9th passage; C) 21st passage; D) 23rd passage. Oligonucleotides that by comparison with the original clone are not present in the mutagenized virus passages are indicated by *stars*, new oligonucleotides are indicated by *arrows* (*Clewley* and *Bishop* 1979). The first dimensions were run from *left to right*, the second from *bottom to top*. The *lower halves* of the patterns contain the unique large oligonucleotides

that were similarly cloned and passaged in the absence of mutagen remained like the zero passage virus (*Clewley* and *Bishop* 1979).

Virus isolates obtained from short-lived epidemics usually give identical fingerprints unless two or more different virus strains are circulating together. In a Western equine encephalitis virus epidemic in North Dakota in 1975, D. Trent (Center for Disease Control, Fort Collins, Colorado) recovered genotypic variants (with one or two oligonucleotide differences) at a frequency of approximately one per fifty isolates analyzed (*D. Trent*, personal communication). Antigenically identical viruses obtained over a period of time usually exhibit identical or almost identical fingerprints. This is true both for viruses obtained from spreading epidemics (e.g., influenza as it spreads throughout a continent over a period of time) as well as for viruses that are endemic

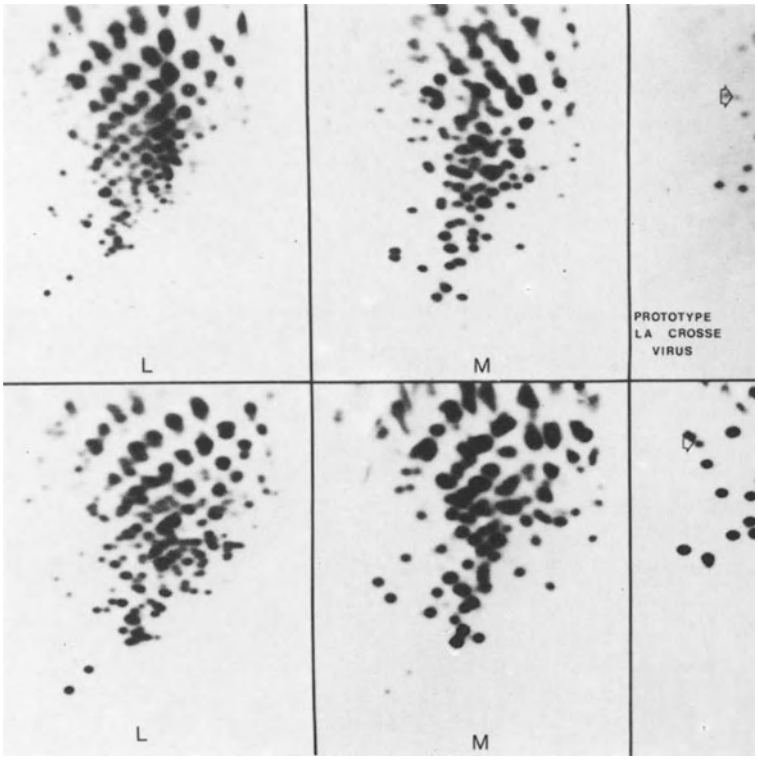

Fig. 2. Oligonucleotide fingerprint analyses of prototype LAC virus (Dresbach, Minnesota, 1960) and a LAC virus isolate obtained from De Soto, Wisconsin in 1978. The oligonucleotide fingerprints of ribonuclease T1 digests of the ^{32}P-labeled L, M, and S RNA species extracted from purified preparations of virus are shown. *Arrows* show identity of S RNA patterns. (*Klimas* et al. 1981)

to particular geographic locations and are recovered over a period of years. An example is shown in Fig. 2, in which the fingerprints of the three RNA segments (L, M, and S) of the La Crosse (LAC) bunyavirus obtained in 1960 from a fatal human infection are compared with those of a second isolate recovered from a second fatal human infection that occurred in 1978. The S RNA patterns of these two viruses are essentially identical, whereas the L (or M) RNA patterns, although similar, are easily distinguished.

La Crosse virus is endemic in particular parts of the United States. The virus is transmitted by certain types of mosquitoes which are limited in their geographic distribution. By analyzing some twenty LAC virus isolates obtained from 1960 to 1978 from various parts of the midwestern, southern, and eastern regions of the continental United States (see Fig. 3), it has been possible to categorize the antigenically identical isolates into both genotypic and geographic groups based on their fingerprint patterns (*Klimas* et al. 1981). The distribution of such groups is exemplified in Fig. 4. The data also show that in some places different genotypic variants of LAC virus are sympatric in nature. The fact that this has permitted LAC virus ressortment to occur has been confirmed by the isolation of recombinant LAC viruses from that region of the United States (*Klimas* et al. 1981).

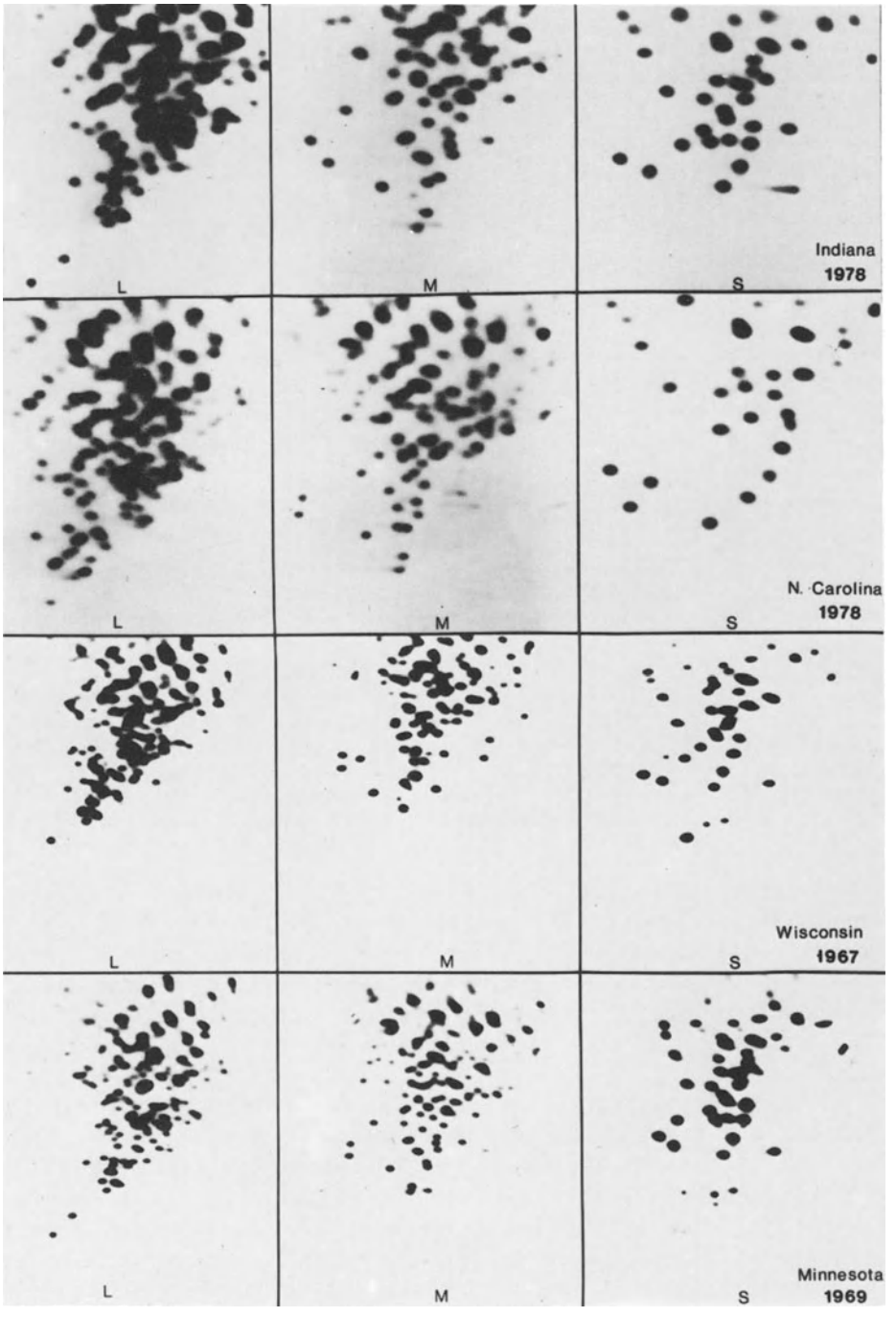

Fig. 3. Oligonucleotide fingerprint analyses of the genomes of LAC 79-264 (Muncie, Indiana, 1978), LAC 78V-13191 (Cherokee, North Carolina, 1978), LAC AP-401 (Meadow Valley, Wisconsin, 1967), and LAC MG4-450 (Gilmore Valley, Minnesota, 1969). *L*, *M*, and *S* refer to the respective RNA species. (*Klimas* et al. 1981)

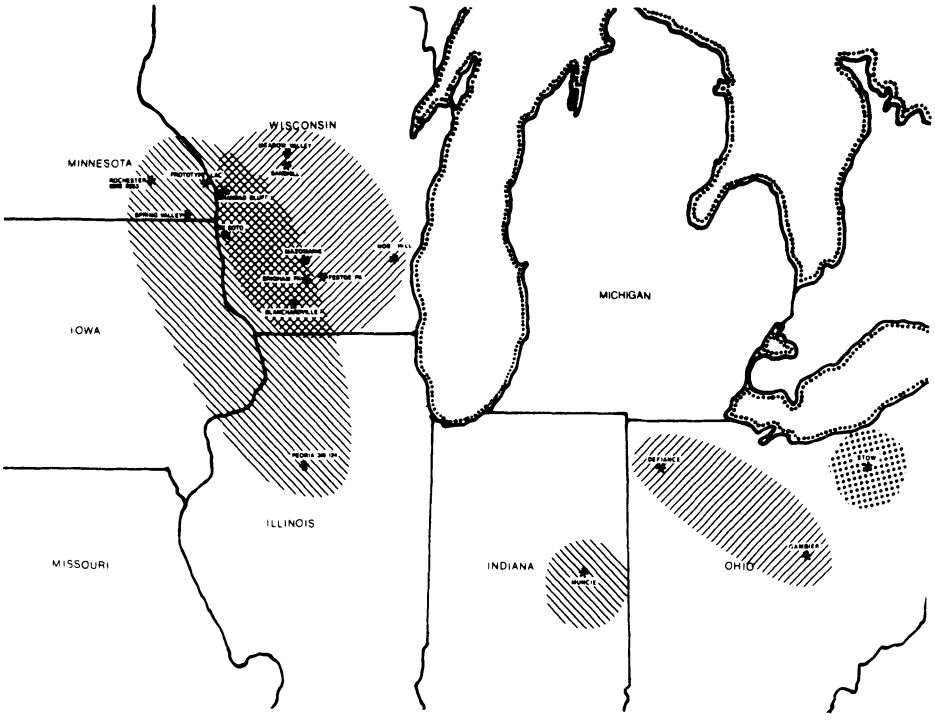

Fig. 4. Geographic distributation of LAC genotypic variants obtained in the upper midwestern United States. The *different types of shaded areas* indicate the regions in the United States from which particular genotypic variants of LAC virus have been obtained. (*Klimas* et al. 1981)

In summary, fingerprinting is useful for characterizing genotypic variants of viruses, monitoring their evolution with the passage of time, and delineating their geographic limits. The limitation of the method is that it does not allow useful comparisons to be made between different virus serotypes, subtypes, varieties, or even some antigenic variants. An example of the inability of the procedure to relate antigenic variants is shown in Fig. 5. Snowshoe hare (SSH) virus is considered a serological variant of LAC virus (*Bishop* and *Shope* 1979). Using particular sera (e.g., one-inoculation guinea pig sera) SSH virus can be distinguished from LAC virus by, for example, kinetics-of-neutralization assays; however, it is not readily distinguished by more standard serological tests (e.g., complement fixation). As shown in Fig. 5, the L, M, and S RNA fingerprints of LAC and SSH viruses do not exhibit sufficient homologies to demonstrate that the viruses are in fact related. Sequence studies, however, provide that proof. The same holds true for alternative serotypes, subtypes, and varieties of the California encephalitis serogroup to which both LAC and SSH viruses are assigned (*Bishop* and *Shope* 1979; *Ushijima* et al. 1980). Thus fingerprinting, although useful for proving the relationships of genotypic variants, is not always useful in relating viruses that are antigenically distinct, even for viruses that are considered as serological variants.

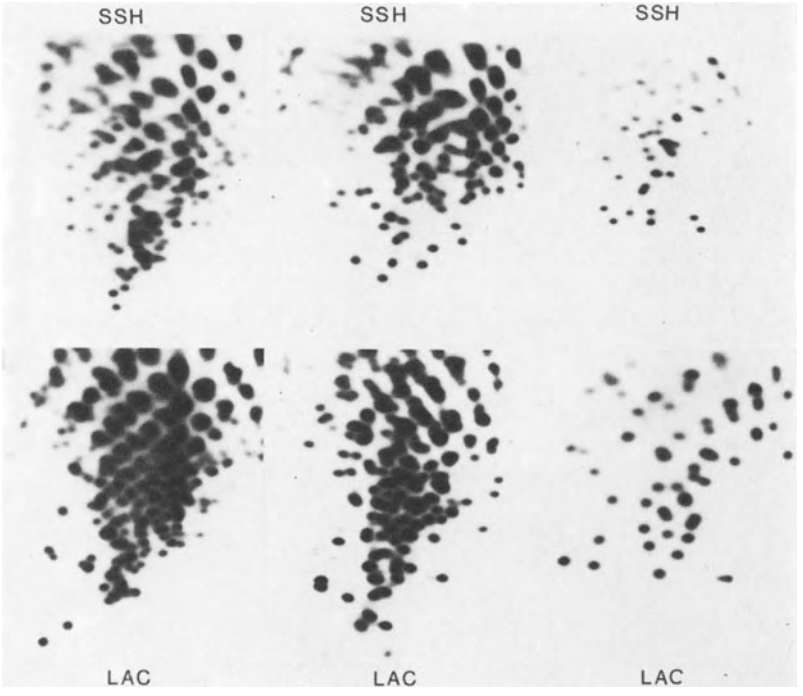

Fig. 5. Oligonucleotide fingerprints of the L, M, and S RNA species (*left to right*) of SSH (*top three panels*) and LAC viruses (*bottom three panels*)

3 Sequencing the Genomes of RNA Viruses

Complete sequence information is currently only available for selected oncornaviruses, orthomyxoviruses, and picornaviruses. Partial sequence data for various RNA viruses have been reported. The terminal sequences of some RNA viruses have been determined by 3′ (or 5′) end labeling of the RNA followed by base-specific enzymatic or chemical cleavage and resolution of the products by polyacrylamide gel electrophoresis. Proximal sequences to the 3′ end of an RNA have been obtained for various viruses by primer-directed copying of the RNA using reverse transcriptase. A method of choice is to use a 3′-complementary oligonucleotide and specifically anneal it to the end of the RNA. Then, using dideoxynucleotides in addition to deoxynucleotides as substrates for DNA synthesis by reverse transcriptase, the chain-terminated products (obtained when dideoxynucleotides are incorporated into the growing chain) are resolved by electrophoresis and their position in the DNA deduced. Using such techniques, the first 200 nucleotides of the S (Fig. 6), M, and L RNA species of SSH, LAC, and an alternative LAC isolate (L74) have been obtained (*Clerx-van Haaster* et al. 1982). For an RNA with the sequence A–Z, one strategy that can be employed to obtain the complete sequence is to use a complementary oligonucleotide primer (A′B′) and by dideoxy-sequencing obtain the sequence through, say, position H. From the information obtained a second oligonucleotide (F′G′) can be synthesized and used to obtain further sequence data. By further sequential analyses the complete RNA se-

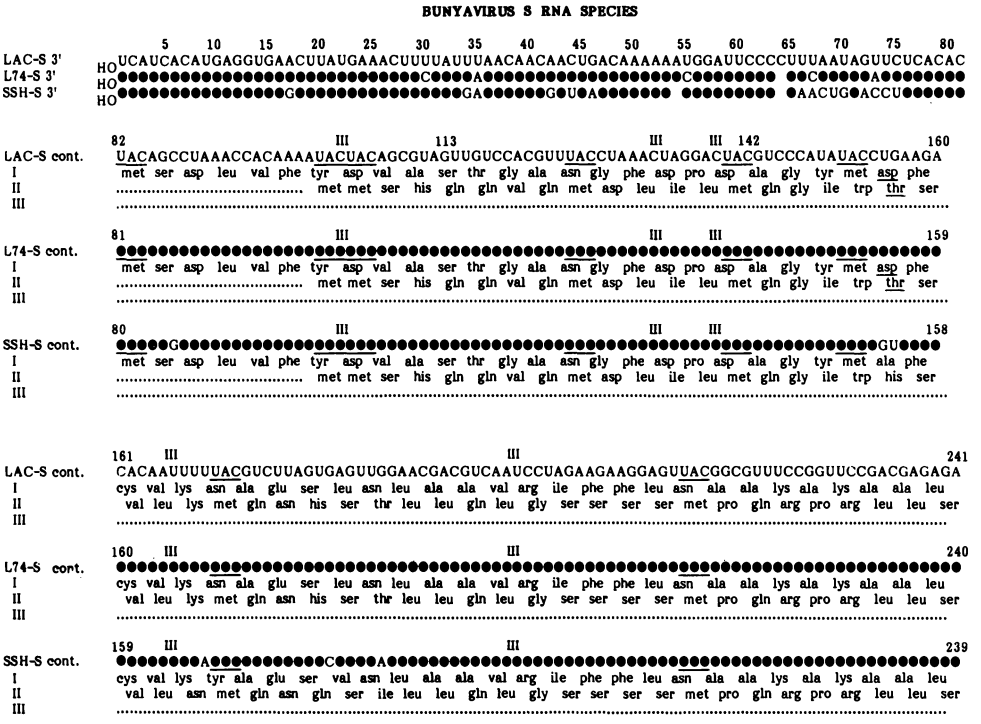

Fig. 6. Deduced prototype LAC virus, prototype SSH virus, and alternate LAC virus (L74) S RNA 3′-terminal sequences and predicted S RNA-encoded gene products. The RNA sequences were obtained from analyses of [^{32}P]pCp-end-labeled S RNA preparations of LAC, an alternate LAC isolate (L74), and SSH viruses. Sequences were also obtained by dideoxynucleotide DNA chain termination analyses with a 3′ terminal complementary oligonucleotide (*Clerx-van Haaster* et al. 1982). The nucleotides in the SSH and L74 viral S RNA sequences that were identical to those in the LAC virus sequences are indicated by *solid circles*. UAC triplets (possible AUG translation initiation codons on the complementary RNA) are *underlined*. After the putative translation initiation sites, the amino acids that were coded in the same reading frame (reading frame, I, II, or III) are shown under the corresponding triplets. The positions of the translation stop signals (antiopal ACU, antiochre AUU, and antiamber AUC) are indicated by the superscripts *I, II,* and *III*, corresponding to the reading frames in which they occur. *Gaps* in the SSH and L74 viral RNA sequences represent missing nucleotides compared with the prototype LAC virus S RNA sequence

quence can be obtained. Such a procedure has been applied to deducing part of the SSH S RNA sequence (*Bishop* et al. 1982). A more common method for obtaining a complete RNA sequence is to clone a DNA copy of the RNA into a bacterial plasmid and then sequence the cloned DNA by standard procedures. Both this and alternative procedures have been used to deduce the sequences of all eight segments of influenza A/PR8/34 (*Fields* and *Winter* 1982; *Winter* and *Fields* 1980, 1981, 1982; *Winter* et al. 1981a, b; *Fields* et al. 1981). The complete sequence of a cloned DNA copy of the S RNA of SSH virus has similarly been obtained (*Bishop* et al. 1982).

The data provided by sequencing are much more informative concerning virus relationships than that provided by fingerprinting. For example, the sequences in Fig. 6 indicate that the S RNA species of LAC and L74 viruses are almost identical;

Table 1. 3′ End sequences of bunyavirus L, M, and S RNA species[a]

Virus		L RNA																					
		1																					22
LAC	3′	U	C	A	U	C	A	C	A	U	G	A	G	G	A	U	A	G	A	U	G	U	U
L74		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
SSH		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	A	•	•
BOR		•	•	•	•	•	•	•	•	•	•	G	•	•	•	•	•	•	•	•	U	•	•
TUR		•	•	•	•	•	•	•	•	•	•	•	•	•	G	A	•	U	G	•	U	•	•
AINO		•	•	•	•	•	•	•	•	•	•	G	•	•	•	•	C	U	C	•	U	•	•
ORI		•	•	•	•	•	•	•	U	•	•	G	C	N	•	C	N	N	•	•	A	A	A
CAP		•	•	•	•	•	•	•	•	•	•	G	•	A	•	C	U	•	U	A	U	•	•
		M RNA																					
		1																					22
LAC	3′	U	C	A	U	C	A	C	A	U	G	A	U	G	G	U	U	C	A	U	A	U	C
L74		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
SSH		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
BOR		•	•	•	•	•	•	•	•	•	•	•	•	•	A	A	C	A	U	•	U	•	•
TUR		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	C	A	U	A	U	•	U
AINO		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	G	U	U	•	G	•	U
ORI		•	•	•	•	•	•	•	U	•	•	G	N	•	A	C	N	N	N	N	N	N	N
CAP		•	•	•	•	•	•	•	U	•	•	G	G	•	A	C	A	G	•	A	•	•	•
MD		•	•	•	•	•	•	•	•	•	•	•	•	•	•	G	•	U	U	A	U	•	U
		S RNA																					
		1																					22
LAC	3′	U	C	A	U	C	A	C	A	U	G	A	G	G	U	G	A	A	C	U	U	A	U
L74		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
SSH		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	G	•	•	•	•	•
BOR		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	G	•	•	U	C
TUR		•	•	•	•	•	•	•	•	•	•	•	•	•	N	N	N	N	N	N	N	N	N
AINO		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	U	A	•	C	U	•
ORI		•	•	•	•	•	•	•	U	•	•	•	A	•	A	A	U	C	•	•	•	C	A
CAP		•	•	•	•	•	•	•	U	•	•	•	A	•	C	A	U	C	•	•	•	C	A
MD		•	•	•	•	•	•	•	•	•	•	•	•	•	•	U	U	U	A	•	•	U	•
MER		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	U	U	A	A	G	U	•
PAH		•	•	•	•	•	•	A	•	•	•	•	A	•	A	U	U	U	•	•	•	U	N

[a] The 3′ end sequences of the L, M, and S RNA species of LAC, SSH, L74, Boraceia (BOR), Turlock (TUR), Aino (AINO), Oriboca (ORI), Capim (CAP), Main Drain (MD), Mermet (MER), and Pahayokee (PAH) bunyaviruses (*Bishop* and *Shope* 1979) were determined as described elsewhere (*Clerx-van Haaster* and *Bishop* 1980). Identical nucleotides to the LAC sequences are indicated by filled circles, unidentified nucleotides by N

Table 2. 3′ End sequences of nairovirus L, M, and S RNA species[a]

Virus		L RNA														
		1														15
QYB	3′	A	G	A	G	A	U	U	C	U	U	U	A	A	U	U
BDA		•	•	•	•	•	•	•	•	•	C	•	•	•	•	•
HUG		•	•	•	•	U	•	•	•	•	•	•	N	N	N	N
AVA		•	•	•	•	U	•	•	•	•	•	•	N	N	N	N
HAZ		•	•	•	•	U	•	•	•	•	N	•	N	N	G	A
DUG		•	•	•	•	U	•	•	•	•	N	•	•	U	G	G
AM		•	•	•	•	U	•	•	•	A	•	•	•	U	A	•
		M RNA														
		1														15
QYB	3′	A	G	A	G	A	U	U	C	U	U	U	A	U	G	A
BDA		•	•	•	•	•	•	C	•	C	C	•	•	N	•	•
HUG		•	•	•	•	U	•	•	•	•	•	•	N	•	•	•
AVA		•	•	•	•	U	•	•	•	•	•	•	G	N	N	N
HAZ		•	•	•	•	U	•	•	•	•	N	•	N	N	•	N
DUG		•	•	•	•	U	•	•	•	•	G	•	U	•	•	C
AM		•	•	•	•	U	•	•	•	A	N	•	N	•	N	N
		S RNA														
		1														15
QYB	3′	A	G	A	G	A	U	U	C	U	G	C	C	U	G	C
BDA		•	•	•	•	•	C	N	•	C	•	U	•	•	•	•
HUG		•	•	•	•	U	•	•	•	•	U	U	U	•	•	•
AVA		•	•	•	•	U	•	•	•	•	U	U	G	•	•	•
HAZ		•	•	•	•	U	•	•	•	•	N	U	U	•	N	N
DUG		•	•	•	•	U	•	•	•	•	•	U	U	•	•	•
AM		•	•	•	•	U	•	•	•	A	U	U	A	•	U	U

[a] The 3′ end sequences of the L, M, and S RNA species of Qalyub (QYB), Bandia (BDA), Hughes (HUG), Avalon (AVA), Hazara (HAZ), Dugbe (DUG), and Abu Mina (AM) nairoviruses (*Clerx* et al. 1981) were determined as described elsewhere (*Clerx-van Haaster* and *Bishop* 1980). Identical nucleotides to the QYB sequences are indicated by filled circles, unidentified nucleotides by N

also, the SSH S RNA sequence is very similar. Close examination of the sequences bounded by G residues for the first 240 residues of LAC and SSH S RNA species (Fig. 6) show that only one of seven large oligonucleotides greater than 12 nucleotides in length (LAC residues 123–136) is shared by SSH virus. By comparison, four of the seven large LAC oligonucleotides are shared by the L74 isolate, a virus which by oligonucleotide fingerprinting has been categorized as a genotypic variant of prototype LAC virus (*Klimas* et al. 1981).

In addition to demonstrating virus relationships within a serotype, sequence data can provide proof of conserved relationships between different virus serotypes assigned to different serogroups of a viral genus. Examples are shown in Tables 1 and 2, in which 3′ end sequences of several bunyaviruses belonging to different serogroups of the *Bunyavirus* genus (*Bunyaviridae*) and several nairoviruses of the *Nairovirus* genus (*Bunyaviridae*) are compared.

4 Conclusions

Sequence data provide information concerning virus relationships that are not always evident from fingerprint analyses. However, obtaining complete or even partial sequence information is both time consuming and labor intensive. Fingerprinting is useful for demonstrating virus relationships at the genome level but is only applicable to characterizing genotypic variants of known viruses. Although RNA fingerprints are relatively easily obtained, their use in viral diagnosis is limited by such constraints until such time as all known virus serotypes and their subtypes (or variants) have been fingerprinted. When this has occurred, fingerprinting will be particularly useful for characterizing virus evolution and determining the geographic distribution of viruses.

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Restriction Endonuclease Patterns of Herpes Simplex Virus DNA: Application to Diagnosis and Molecular Epidemiology

BERNARD ROIZMAN* AND MAURO TOGNON**

1 Introduction

Historically, the indications that herpes simplex virus (HSV) strains may vary considerably emerged many years ago (*Ashe* and *Scherp* 1965; *Hampar* and *Burroughs* 1969). For one of us (BR), the studies on the variability of HSV began with a chance isolation of a mutant [HSV-1(MP)] that fused cells into polykaryocytes from a wild type virus [HSV-1(mP)] which caused cells to round and clump (*Hoggan* and *Roizman* 1959). Subsequent studies showed that the parent and mutant differed with respect to buoyant density in CsCl gradients (*Roizman* and *Roane* 1961), affinity chromatography (*Roizman* and *Roane* 1963), neutralization with specific antisera (*Roizman* and *Roane* 1963), the ability to accumulate glycoprotein VP8(gC) (*Heine* et al. 1974) and, ultimately, with respect to several genetic loci concerned with cell fusion in addition to accumulation of glycoprotein VP8(gC) (*Ruyechan* et al. 1979). The discovery that HSV represents two viruses, HSV-1 and HSV-2 (*Schneweiss* 1962; *Dowdle* et al. 1967), and studies on isolates and mutants of each (*Ejercito* et al. 1968; *Heine* et al. 1974; *Seth* et al. 1974; *Terni* and *Roizman* 1970), reinforced the hypothesis that HSV strains vary in a number of characteristics.

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Our interest in the diversity of HSV stemmed from the realization that these viruses are responsible for a wide repertoire of diseases. In an attempt to determine whether HSV strains differing with respect to site of localization in the human body or pattern of disease also differed with respect to structural proteins, we discovered that although a correlation between clinical manifestations and structural proteins could not be demonstrated, the variability in the electrophoretic mobility of several of the structural proteins was beyond our expectations (*Pereira* et al. 1976). The study made apparent the now established fact that the HSV genomes are variable; perhaps more important, the identity of viruses isolated from epidemiologically related individuals suggested that the electrophoretic patterns of the structural proteins could be used to identify individual viruses and follow their spread in the human population. Fortunately, while these studies were readied for publication, it became apparent that a similar variability could be demonstrated by electrophoretic separation of restriction enzyme digests of viral DNAs at a fraction of the cost of protein analyses (*Hayward* et al. 1975).

This paper is concerned with the variability in HSV genomes and the use to which this variability can be put to answer specific questions regarding the epidemiology of herpesviruses in human populations. Fundamentally, the variability in HSV genomes may be summarized as follows:

1. HSV-1 and HSV-2 DNAs differ in base composition (*Goodheart* et al. 1968; *Kieff* et al. 1971). Although there is considerable homology with good matching of base pairs (*Kieff* et al. 1972), the restriction enzyme cleavage sites mapped to date do not correspond (*Morse* et al. 1977; *Marsden* et al. 1978). Because the electrophoretic patterns of HSV-1 and HSV-2 DNA digests are vastly different, they can be used to differentiate between the two subtypes of HSV (*Skare* et al. 1975; *Hayward* et al. 1974).

2. Epidemiologically unrelated strains of HSV-1 and HSV-2 appear to differ with respect to the presence of restriction endonuclease cleavage sites (*Linneman* et al. 1978; *Buchman* et al. 1978, 1979, 1980; *Halperin* et al. 1980; *Hammer* et al. 1980; *Lonsdale* et al. 1979; *Whitley* et al., to be published). These properties of the HSV genomes appear to be stable and can be used to trace the epidemiology of the virus in human populations. The variability in restriction enzyme cleavage sites does not obscure the inherent differences between HSV-1 and HSV-2 DNAs.

3. Certain regions of the HSV-1 and HSV-2 genomes appear to vary in size because of amplification and reduction in sequences at the termini of the genome and at other specific sites (*Hayward* et al. 1975; *Wagner* and *Summers* 1978; *Locker* and *Frenkel* 1979; *M. Tognon* and *B. Roizman*, manuscript in preparation). Because DNA fragments containing these regions of the genome may vary on serial propagation of the virus they should be identified and not used for specific differentiation of HSV isolates in epidemiologic studies (*Roizman* and *Tognon* 1982).

2 Variability in Restriction Enzyme Cleavage Sites

The results presented in this section center on two well-documented conclusions cited earlier in the text that HSV-1 and HSV-2 DNAs vary with respect to the presence or

absence of restriction endonuclease cleavage sites, and that these differences do not obscure the lack of correspondence of cleavage sites in the DNAs of these two subtypes of HSV.

The lack of correspondence between the mapped restriction endonuclease cleavage sites in HSV-1 and HSV-2 DNAs emerge from examination of the maps of colinear arrangements of the two DNAs shown in Fig. 1. This figure also shows some of the variable restriction endonuclease cleavage sites observed in our laboratory with all enzymes except *Bam*HI, *Kpn*I, and *Sal*I. Given the differences in the number of cleavage sites and the sizes of the fragments, it can be readily expected that HSV-1 and HSV-2 DNAs could be differentiated from each other even if all of the variable cleavage sites noted for any one enzyme shown in this figure were to vary at once.

The variable cleavage sites noted in Fig. 1 were determined from analyses of the DNAs of more than 120 HSV-1 and 50 HSV-2 isolates with *Xba*I, *Hsu*I (*Hin*dIII isoschizomer), *Bgl*II, *Hpa*I, and *Eco*RI restriction endonucleases. The variability observed with *Bam*HI, and *Kpn*I restriction endonucleases after 1979 (*Locker* and *Frenkel* 1979) was also very extensive, and because of the large number of fragments generated by each enzyme, it was never tabulated.

Several comments should be noted in connection with the variability in restriction enzyme cleavage sites observed to date:

1. The restriction enzyme cleavage pattern of the DNA of any given isolate appears to be quite stable. This conclusion is based chiefly on two studies. First, HSV-1(MP) propagated serially at least 80 times between 1958 and 1968, when the practice of serially propagating viruses was terminated, could not be differentiated from either the second serial passage or a concurrently, examined high-passage stock (1968) of the parent, HSV-1(mP) virus. The second study was stimulated by the observation that the HSV-1(KOS) strain obtained from Dr. P.A. Schaffer did not correspond with respect to restriction enzyme cleavage sites to the KOS strain obtained from Dr. F. Rapp. Subsequent comparisons with a fresh isolate from the same individual established that the virus obtained from Dr. Rapp could not be differentiated from the isolate obtained from the same patient 12 years later.

2. Variation in the presence or absence of restriction endonuclease cleavage sites reflects spontaneous insertions, deletions, and substitutions of bases at nonlethal sites in HSV DNA. A recent conference on genetic variation of viruses (*Palese* and *Roizman* 1980) made it apparent that variation of the type observed with HSV DNAs has also been observed with a variety of other DNA and RNA viruses. Although most of the epidemiologic studies carried out to date involved enzymes which recognize six nucleotides, a single base-pair deletion, insertion, or substitution would siffice to preclude cleavage. Such mutations probably occur in human infection at a slow rate; they are perpetuated and disseminated when the mutated virus is transmitted from one individual to another. The observed variability in HSV DNA probably reflects the millenia of association of the virus with the human population. The apparent stability of the viral DNA probably reflects the test conditions rather than a moratorium on mutations. Specifically, it is likely that people are infected with relatively low doses of virus and hence the progeny of the infecting virus, and hence the pool available for transmission to other people, are clonally related. Because mutant viruses generated after infection would represent a small fraction of the total pool, the probability of

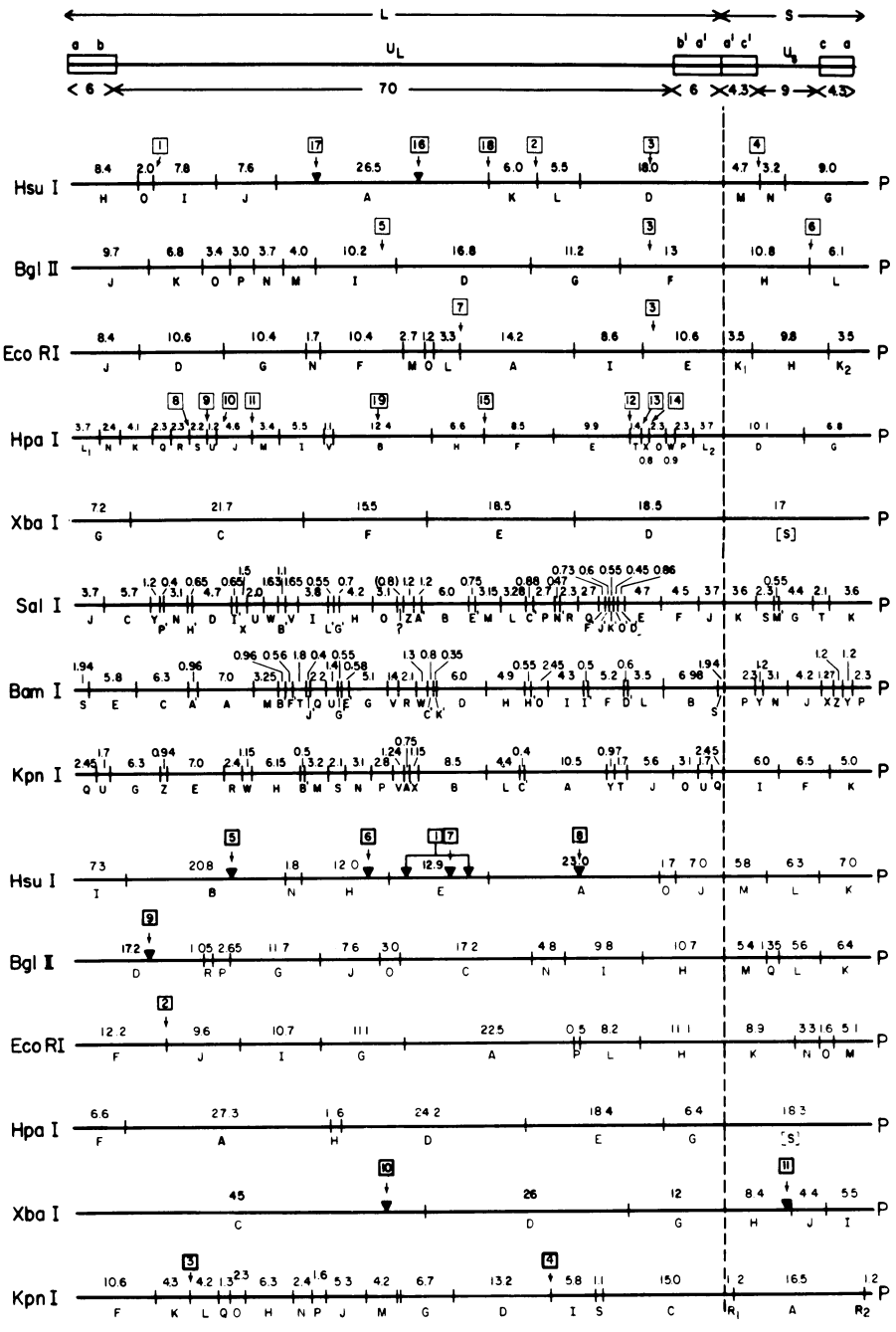

Fig. 1. Sequence arrangements and restriction endonuclease maps of HSV-1(F) and HSV-2(G) DNAs. The sequence arrangement in HSV DNA is shown in the *top line* according to *Wadsworth* et al. (1975). The *numbers* below the line are percent of total DNA. The next seven lines represent the restriction enzyme maps for HSV-1(F), whereas the last five lines represent HSV-2(G) maps. For derivation of maps see *Roizman* (1979). The *numbers within squares* above the line represent restriction endonuclease sites either absent (*light arrow*) from DNAs of strains other than HSV-1(F) and HSV-2(G) or present in other strains but absent from HSV-1(F) or HSV-2(G) DNAs (*heavy arrow*). The exception is *3* which represents a deletion in HSV-1(HFEM) DNA. The variability in *Kpn*I and *Bam*HI cleavage sites is not shown

transmission to another person is small and reflects the fraction of the mutants in the pool. In addition, the total number of HSV-1 cleavage sites tested with *Xba*I, *Kpn*I, *Bam*HI, *Eco*RI, *Hin*dIII, *Bgl*II, and *Hpa*I is of the order of 160 and involves approximately 1000 base pairs. Since HSV DNA has approximately 160000 base pairs, the probability that a mutation would occur in one of the 1000 base pairs being tested and that the particular mutant rather than the parent would be transmitted from one individual to another is rather slim. Nevertheless, transmission of mutated viruses is likely to occur and be documented, especially as the fraction of the genome being sampled with restriction endonucleases increases.

3. Because the distribution of restriction enzyme cleavage sites is obviously nonrandom, the question arises whether some restriction sites are more likely to accumulate mutations than others by virtue of being located in regions of the genome which tolerate changes in base composition. Our impression is that *Hin*dIII (*Hsu*I) cleavage sites were much more variable than *Eco*RI and *Bgl*II sites for both HSV-1 and HSV-2 DNAs. However, whereas *Hpa*I cleavage sites in HSV-1 DNA were highly variable, the variability of *Hpa*I sites in HSV-2 DNA was negligible.

3 Generation of Variability in HSV Genomes by Sequence Accretion and Deletion

The alterations in the HSV genomes due to sequence accretion and deletion are confined to two specific regions of the genome. Although it is convenient to discuss the two regions separately, it is desirable to precede the discussion with a brief review of the overall structure of HSV DNA.

3.1 The Sequence Arrangement in HSV DNA

Sheldrick and *Berthelot* (1975) were the first to report that the termini of HSV-1 DNA are inverted internally, separating the genome into a long (L) and a short (S) component. They suggested that the two components may invert by recombination of the termini with their inverted repeats. Subsequent studies have shown that:

1. The sequences flanking the L component designated ab and b′a differ, except for the a sequence, from the sequences flanking the S component and designated a′c′ and ca (*Wadsworth* et al. 1975).

2. The L and S components invert relative to each other; cells infected with wild type viruses accumulate and package four populations of DNA molecules differing solely in the orientation of L and S components (*Roizman* et al. 1974; *Hayward* et al. 1975; see review by *Roizman* et al. 1979).

3. The inversions in the HSV DNA appear to be due to a specific sequence – the a sequence – in as much as the insertion into the L component of an additional a sequences, causes additional inversions to occur (*Mocarski* et al. 1980; *Mocarski* and *Roizman* 1981).

A schematic diagram of the sequence arrangement in HSV is shown in Fig. 1. It should be noted that enzymes which do not cut within the ab, b′a′, a′c′, and ca se-

quences produce three sets of fragments: (a) four terminal fragments each present in 0.5-*M* concentration relative to the molarity of intact DNA, (b) four fragments spanning the junction between the L and S components each present in 0.5-*M* concentration relative to that of intact DNA, and (c) a variable number, depending on the number of cleavage sites, of fragments equimolar to intact DNA (*Hayward* et al. 1975). Until the structure of HSV DNA became apparent, the submolar fragments suggested heterogeneity in HSV DNA sequences.

3.2 Variability in HSV DNA Termini

Several laboratories, but notably *Hayward* et al. (1975) and *Skare* et al. (1975), noted that the terminal DNA fragments vary with respect to electrophoretic mobility. More recent studies have shown this variability is due to amplification or reduction in the number of terminal a sequences (*Wagner* and *Summers* 1978; *Locker* and *Frenkel* 1979). Specifically, viral DNA may contain several copies of the a sequence at the junction of the L and S components and at either the L terminus or the S terminus (*Wagner* and *Summers* 1978; *Locker* and *Frenkel* 1979). The terminus, L (e.g., HSV-1 (F); *Locker* and *Frenkel* 1979) or S (e.g., HSV-1 (Angelotti); *Kaerner* et al. 1981), that contains the amplified a sequence is genetically determined by the virus. The abundance of junctions or termini with more than three a sequences is relatively low, but as many as 14 copies of the a sequences were reported in derivatives of HSV-1 (Angelotti) DNA (*Kaerner* et al. 1981). The number of a sequences can be amplified or reduced as evidenced by the observation that the progeny of plaque-purified virus show the entire spectrum of variability in the number of a sequences. The variability in the number of a sequences is particularly apparent and can be mistaken for presence or absence of a cleavage site in electrophoretic patterns of HSV DNA cleaved with an enzyme which makes numerous cuts (e.g., *Bam*HI, *Sal*I, and *Kpn*I).

3.3 Variability due to Accretion or Deletion of Sequences Other than the a Sequence

Variability in the electrophoretic migration of nonterminal fragments was noted by *Hayward* et al. (1975), *Skare* et al. (1975), *Wagner* and *Summers* (1978), and *Locker* and *Frenkel* (1979). Recent studies on these regions by *M. Tognon* and *B. Roizman* (manuscript in preparation) may be summarized as follows:

1. Analyses of clones of plaque-purified stocks of several viruses with *Bam*HI and *Kpn*I revealed that several fragments varied in electrophoretic mobility. Of particular interest were several nonterminal fragments (Fig. 2, e.g., *Bam*HI B, *Bam*HI E, *Kpn*I J, etc.). These fragments map near the termini of HSV DNA, but this variability in HSV DNA appears to be unrelated to the a sequences.

2. In order to determine whether the variation in electrophoretic mobility was unidirectional, i.e., from slow to fast, or bidirectional, i.e., from slow to fast and vice versa, plaque-purified variants showing rapidly and slowly migrating fragments were plaque purified a further three times. Individual plaques were then picked and virus stocks were prepared. Restriction endonuclease analyses of these stocks (Fig. 3) showed that the fragments can vary from fast- to slow-migrating and vice versa and

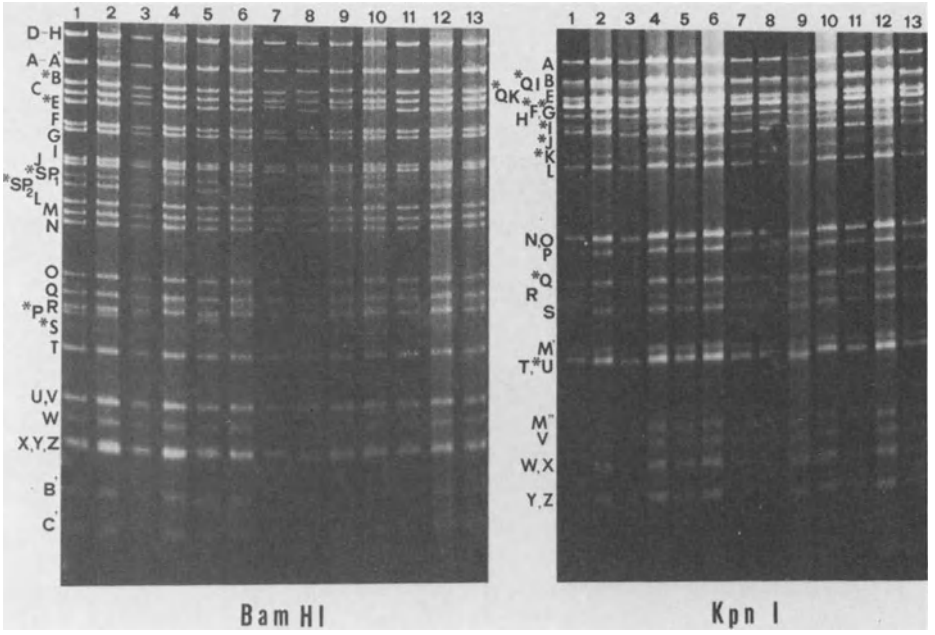

Fig. 2. *Bam*HI and *Kpn*I restriction endonuclease profiles of the DNAs of plaque-purified clones of HSV-1(mP) virus. In HSV-1(mP) DNA, *S*, *P*, and *SP* represent the *Bam*HI terminal and junction fragments, whereas *Q*, *I*, *K*, *QI*, and *QK* are the *Kpn*I terminal and junction fragments. The fragments showing heterogeneity in the number of a sequences are *Bam*HI *S* and *SP* and *Kpn*I *Q*, *QI*, and *QK*. The *double letters* identify L-S junction fragments. Fragments with the *subscript* 1 or 2 identify terminal or junction fragments with one or two a sequences, respectively. The *asterisks* identify all fragments which vary in electrophoretic mobility

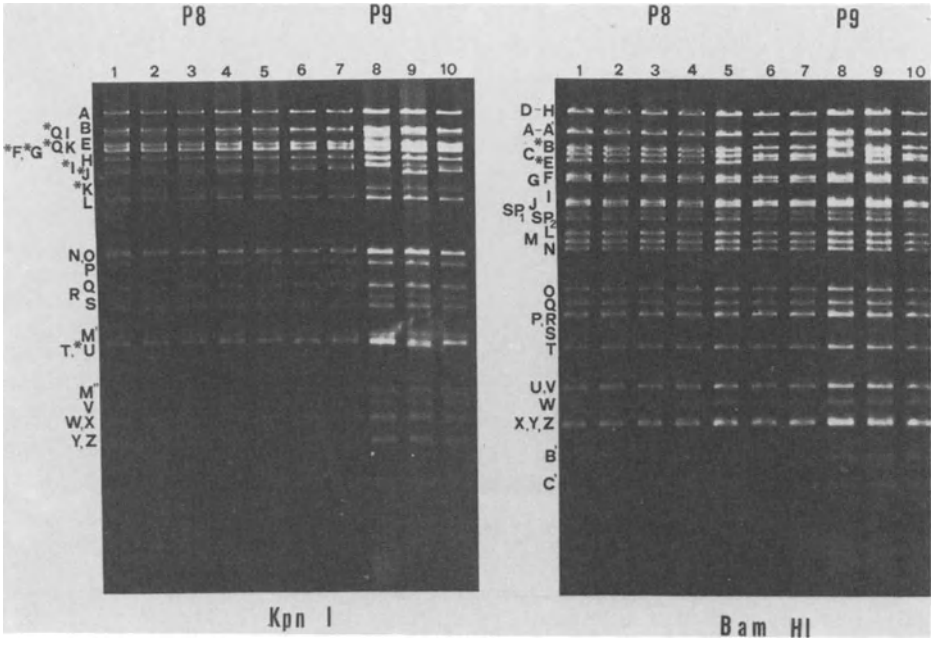

Fig. 3. Restriction endonuclease patterns of HSV-1(mP) DNAs from progeny of plaque-purified populations derived from plaques *8* and *9* of Fig. 2. *1–5* were derived from progeny of *8*, whereas *6–10* were derived from progeny of *9*. Lettering as defined for Fig. 2

suggest that sequences contained in these fragments can undergo accretion or deletion by either unequal crossing over, slippage, or by sequence insertion.

Although the variability in electrophoretic mobility suggests that the increase or decrease in nucleotide sequence probably does not exceed 200 base pairs, the variability can be ascribed to a cleavage which generates an additional fragment too small to be detected by electrophoresis. Variations in electrophoretic mobility due to accretion or deletion of sequences in terminal or subterminal fragments of the type described here should not be used to establish nonidentity of viral isolates.

4 Application of Restriction Endonuclease Analysis to Clinical Virology

Restriction endonuleases have developed into the most powerful tool yet devised for the study of the structure and function of DNA virus genomes. The application of restriction endonuclease to problems in clinical virology has been slower but its potential is no less significant.

4.1 Diagnosis of Herpesvirus Infections and Identification of Serotype

Although identification of isolates as HSV presents few problems, serotyping of fresh isolates can be time consuming and error prone in the absence of proven type-specific serologic reagents. Our experience with isolates sent to us for identification suggests that the error rate is high and usually reflects the expectation of most laboratories that isolates from the newborn and from urogenital organs of adults should be HSV-2, whereas oro-facial and brain isolates other than those from neonates should be HSV-1. A reflection of this type of mistyping is illustrated in Fig. 4. This figure illustrates the restriction enzyme patterns of the DNAs isolated from three infants who died in a hospital nursery of infection during a 9-week interval and from two patients in the same community unrelated to the infants. These were sent to us to determine whether the isolates from the neonates were identical. Although the figure shows that all the isolates are quite distinct and unrelated, it is evident that all are HSV-1 and not HSV-2, as originally typed by the hospital laboratory

Although at this time the typing of isolates is of no benefit to the patient, it is desirable to establish the incidence of HSV-1 and HSV-2 infections in the population at risk. The available information on incidence may be unreliable as well as changing. Typing of isolates with bouquets of monoclonal antibodies of defined serotype may well be more cost effective but will probably be more fallible than the restriction endonuclease analyses of HSV DNAs.

4.2 Transmission of Virus from Person to Person

In several recent studies, restriction endonuclease analyses of HSV DNAs were done to determine whether simultaneous or overlapping infections in a population at risk were epidemiologically related (*Linneman* et al. 1978; *Buchman* et al. 1978; *Halperin* et al. 1980). The objective of all of these studies was to determine whether viruses isolated

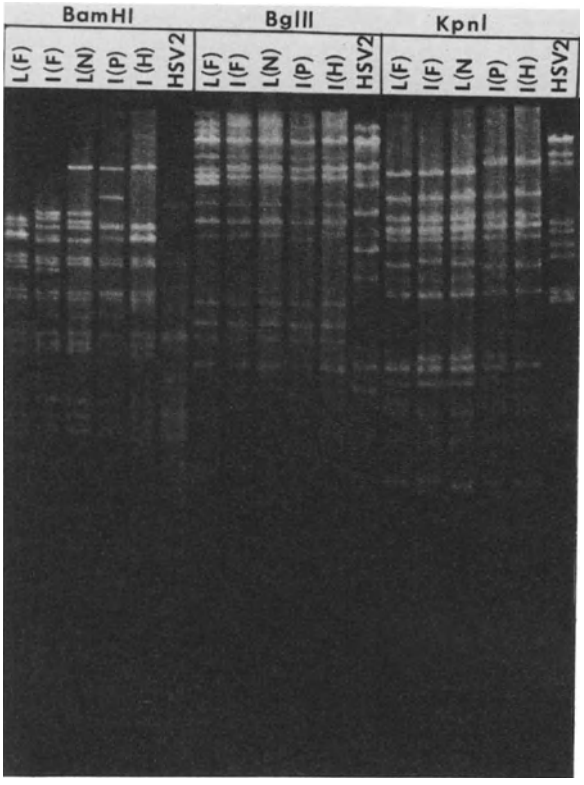

Fig. 4. Restriction endonuclease patterns of the DNAs of HSV-1 isolates. The isolates designated (*I*) were obtained from infants who died of HSV infection in a hospital nursery in a city in the Eastern USA. The isolates marked (*L*) were isolated from patients in the community and are unrelated epidemiologically to the infant isolates. Because the strains were originally serotyped as HSV-2, the DNA of HSV-2(G) was digested and subjected to electrophoresis for comparison

from two or more epidemiologically "connected" individuals were identical. The presumption was that if the viruses infecting these individuals were different, then the infection could not have been caused by transmission of virus from one individual to another. In all of the studies cited above, the laboratory collecting the specimens was asked to include among the viruses to be tested other isolates from the same environment, but not epidemiologically related to the case under study. Furthermore, all specimens were coded, and the code was not broken until the study was completed. In chronological order, these studies (a) failed to differentiate between viruses isolated from two newborns housed in the same pediatric intensive care unit, suggesting person-to-person transmission (Fig. 5A; *Linneman* et al. 1978), (b) demonstrated that viruses isolated from personnel and patients in another pediatric intensive care unit fell into two clusters indicating transmission from infant to personnel in one instance and from personnel to patient and personnel in the second (*Buchman* et al. 1978; *Adams* et al. 1981), and (c) demonstrated that viruses isolated from two newborns in proximity to each other for a period of several hours in a hospital nursery differed by several restriction endonuclease cleavage sites (Fig. 5B; *Halperin* et al. 1980). It should be

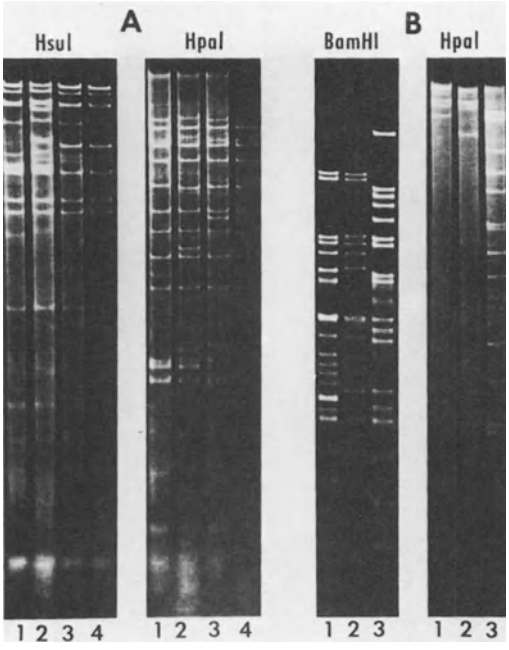

Fig. 5. Electrophoretic patterns of clinical isolates: application of restriction endonuclease analyses to molecular epidemiology. *A*) Electrophoretic patterns of the DNAs from HSV isolated from two infants (*3* and *4*) and two unrelated strains. *Hsu*I is an isoschizomer of *Hin*dIII. Adapted from *Linneman* et al. 1978. *B*) Electrophoretic patterns of DNAs of HSV isolated from two infants (*1* and *2*) and an unrelated strain. Adapted from *Halperin* et al. 1980. Note that in *A* the patterns *3* and *4* are identical, whereas in *B* the patterns *1* and *2* are not identical. The profiles of the DNAs *1* and *2* in *A* differ from each other as well as from *3* and *4*

underscored that although nosocomial aquisition of HSV has been suspected in many instances, the failure to discriminate in blind tests between viruses isolated from epidemiologically related patients in our studies proved its occurrence and indicates the need for (a) screening of pregnant women for genital HSV infection to isolate and preclude transmission to her newborn and others, and (b) training of hospital personnel to preclude self infection as well as transmission of virus from patient to patient. The publicity generated by these cases helped, we think, to institute many desirable changes in the handling of demonstrated or suspected cases of HSV infection among both patients and personnel in hospital units dealing with immunologically compromised patients.

4.3 Susceptibility to Superinfection

A key question regarding the natural biology of HSV is whether individuals infected with HSV-1 or HSV-2 are immune to superinfection with viruses of the same serotype even though they are not immune to superinfection with heterologous viruses. In one series of experiments, *Buchman* et al. (1979) demonstrated that two of eight patients with genital HSV-2 yielded in at least one recurrence a second HSV-2 strain in addition to the one isolated in previous recurrences. Different HSV strains of the same serotype have also been isolated from brain biopsy material and throat washings of patients with encephalitis (*Whitley* et al., to be published). One interpretation of these results is that the "second" virus was introduced into these patients simultaneously with the first virus of the same serotype. The interpretation is plausible but faulty because the person transmitting the virus had to have both viruses. That person, in turn, acquired

the two viruses either sequentially or simultaneously. If simultaneously, than the person infecting him or her had to be infected with both viruses, again either sequentially or simultaneously. At some point in the chain of transmission, one person had to be infected sequentially.

A key question is whether a second, superinfecting virus of the same serotype could colonize the sensory or autonomic ganglia harboring the first infecting virus. *Lonsdale* et al. (1979) reported the isolation of only one virus from any one human ganglion. In experimental studies, *Centifanto-Fitzgerald* et al. (1982) also reported failure to colonize latently infected ganglia with a second virus. The conclusions, if correct, are of considerable significance in as much as it should be possible to construct avirulent viruses capable of colonizing ganglia but which would not be inducible. Such viruses may preempt establishment of latency even though they may not protect from superinfection with wild type viruses of the same serotype. There is a possibility, however, that the experimental design employed to activate the latent virus in ganglia may have precluded a second virus from being activated. The problem stems from the possibility that the first virus colonizing ganglia may infect a large number of neurons and when activated, it may begin multiplying first and may "dilute out" the genomes of any other virus which may be present in the same ganglion. Preliminary studies (*B. Megnier* and *B. Roizman*, work in progress) indicate that different viruses can colonize the same ganglion on sequential infection.

The demonstration that infected individuals may be superinfected with virus of the same serotype augurs poorly for HSV vaccines, unless it can be shown that prior infection reduces the probability of superinfection even though it does not prevent it altogether.

4.4 Variability of the Genomes of Other Herpesviruses

Recent studies summarized by *Dambaugh* et al. (1980) and *Huang* et al. (1980) reported that epidemiologically unrelated isolates of Epstein-Barr and human cytomegalovirus, respectively, vary considerably with respect to restriction enzyme cleavage sites. In the case of human cytomegaloviruses, restriction endonuclease cleavage patterns demonstrated the identity of viruses isolated from a blood donor and a recipient, and nonidentity of viruses isolated sequentially from a family of three children suffering from hemophilia and persistent cytomegalovirus infections. In the latter case, the data suggest that the children were superinfected by a second, different virus which replaced the first.

Recently, several laboratories used restriction endonucleases to differentiate between viruses sharing biological and immunological properties. Thus *Sabine* et al. (1981) and *Studdert* et al. (1981) reported that isolates classified as equine herpesvirus 1 fall into two groups differing with respect to restriction enzyme cleavage sites. Although the number of isolates was small, these studies suggest that epidemiologically unrelated isolates in each group may vary as much as the HSVs. More recent studies show that the two groups of equine viruses may be differentiated by other criteria as well (*Turtinen* et al. 1981). *H. Ludwig* and associates (personal communication) have shown that bovine herpesviruses may also be clustered into subgroups on the basis of restriction endonuclease patterns of their DNAs.

5 Conclusions

Restriction endonuclease analyses of viral DNAs have been successfully applied in a variety of clinical and epidemiologic studies to identify viruses and trace their movement in the human population. The availability of restriction endonucleases, and recent technical developments which obviate the need for radioactive precursors to visualize the electrophoretic patterns of the DNAs, place the test within the reach and competence of most clinical and diagnostic laboratories. The limiting factor, however, is interpretation rather than execution, or availability of reagents to carry out the tests. Interpretation of restriction endonuclease patterns, like that of all other epidemiologic data, requires judgment and knowledge. Failure to differentiate between variability in size due to sequence amplification or reduction or variability in restriction enzyme cleavage sites, may lead to erroneous conclusions of considerable medical and legal importance. Such errors are very likely to occur, especially if the restriction endonuclease map of the virus being analyzed is not readily deduced from the published maps of prototype viruses.

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Nucleic Acid Hybridization for the Detection of Viral Genomes

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1 Introduction

Single-stranded nucleic acids can form stable duplexes if they encounter complementary sequences under appropriate conditions. This reaction is called hybridization and has been extensively used during the past 15 years to explore the structure and expression of cellular and viral genes. Particularly with the advance of molecular cloning, hybridization with specific probes has made it possible to isolate and study individual genes from genomic libraries and has thus contributed significantly to our understanding of eukaryotic gene organization.

Molecular hybridization using radioactively labeled viral nucleic acids can generally be used to detect viral sequences in clinical specimens. In most cases, however, other more simple techniques may be used successfully to demonstrate the presence of viruses and thus eliminate the need to apply nucleic acid hybridization to diagnostic problems. At present, nucleic acid hybridization as a diagnostic tool suffers in that it requires some experience and an advanced technology and is thus hardly applicable to diagnostic routine in many laboratories. However, in some instances hybridization

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has already turned out to be particularly useful to establish the association of a virus with a specific disease. In such instances the detection of viral nucleic acids by molecular hybridization can be used to establish the diagnosis of a specific disease.

2 Theoretical Background

Molecular hybridization is in principal based on the fact that the denaturation of nucleic acids is a reversible process. Double-stranded DNA or RNA is converted into single strands at high temperature and neutral pH. The temperature at which half of the DNA or RNA is into converted single strands is defined as the melting temperature (T_m). It is strongly dependent on the base composition of the DNA (*Marmur* and *Doty* 1962), the monovalent cation concentration (*Schildkraut* and *Lifson* 1965), and the concentration of denaturing agents such as formamide, which are often used to reduce the temperature of hybridization (*McConoughy* et al. 1969). Using Schildkraut's equation in the modification of *Thomas* et al. (1976)

$$T_m = 81.5 + 0.50\,(\%G + \%C) + 16.6 \log Na^+ - 0.60\,(\%\ \text{formamide})$$

the T_m of any given DNA in formamide with known average base composition can be calculated. If, after denaturation, the temperature is lowered to about 20°–30 °C below the T_m, the complementary strands can reanneal to form stable duplexes. The velocity of renaturation follows second order kinetics and is dependent on the concentration of specific nucleic acid sequences which can form duplexes if temperature, salt and formamide concentration, and the size of the DNA fragments are kept constant (*Wetmur* and *Davidson* 1968). If we consider the renaturation of the DNA of a small phage or virus and compare it with the renaturation of the relatively complex genome of *Escherichia coli*, a low concentration of the phage DNA will result in the same molarity of specific sequences as results from a high concentration of *Escherichia coli* DNA. If no repetitive sequences are present in the DNA or RNA, the size of a genome directly reflects its complexity. Analysis of DNA reassociation kinetics has revealed the importance of repetitive sequences for the genome organization of higher organisms (*Britten* and *Kohne* 1968).

3 Hybridization Methods

If a clinical specimen is to be analyzed for the presence of viral nucleic acids, different techniques of nucleic acid hybridization may be applied. Regardless of the technique to be used, DNA has to be extracted from the clinical specimen by conventional means, including lysis of the cells, digestion of proteins with proteinase, and repeated extraction by phenol or phenol-chloroform mixtures. Additionally, the viral DNA or RNA in question has to be available as a labeled hybridization probe. Cloning of viral DNA in a bacterial host provides almost unlimited quantities of the probe and thus allows its distribution to many laboratories. Moreover, the cloning procedure ensures that the sequences amplified in *Escherichia coli* are absolutely free from cellular sequences and thus ensures a high specificity of the probe. A number of different techniques may be used to label the DNA in vitro with radioactive precursors. The

so-called nick translation method, using the repair reaction of *Escherichia coli* DNA polymerase I after the introduction of single-strand nicks by DNase I, has been most widely used and results in specific activities of about 10^8 cpm/μg using ^{32}P-labeled deoxynucleotide triphosphates as precursors (*Rigby* et al. 1977). Hybridization with highly labeled single-stranded probes cloned into the bacteriophage M13 was reported to result in even higher sensitivity (*Faras* and *Messing*, personal communication).

3.1 Reassociation Kinetics

As stated above, the renaturation velocity of a given DNA in solution is dependent on the concentration of specific sequences. If a cellular DNA is to be tested for the presence of viral sequences, trace amounts of radioactively labeled viral DNA are added in order to measure the renaturation velocity of the viral DNA. If the cellular DNA is devoid of viral sequences, the labeled viral probe forms duplexes only at a low rate, reflecting the low concentration of labeled viral tracer DNA. If the cellular DNA contains viral sequences, the labeled tracer DNA forms duplex hybrids at a higher rate. The rate of duplex formation is proportional to the concentration of the respective viral sequences within the cellular DNA and thus allows the amount of viral DNA in the specimen to be quantitated (*Gelb* et al. 1971). Hydroxyapatite chromatography or S1 nuclease digestion, both of which discriminate between single- and double-stranded DNA, can be used to follow the renaturation kinetics. This technique is very sensitive; however, it requires large amounts of DNA (1–2 mg) to detect a single copy or less of a viral genome within a cell. Although this technique has been used widely in the past decade, it is now usually replaced by the techniques described below.

3.2 Filter Hybridization

Single-stranded DNA or RNA can be immobilized on nitrocellulose paper under appropriate conditions without losing the ability to form stable duplexes (*Gillespie* and *Spiegelman* 1965, *Thomas* 1980). If nitrocellulose filters carrying immobilized cellular DNA are incubated with a radioactively labeled viral probe under appropriate conditions, binding of the probe onto the filter can be used to measure the concentration of viral sequences in whole cellular DNA. The binding of the probe to the filter can be measured by autoradiography or by counting the filter in a liquid scintillation counter, or by both tequniques.

Filter hybridization using labeled DNA or complementary RNA, synthesized from a DNA template by *Escherichia coli* RNA polymerase, have been used extensively in the past to search for viral sequences in different tumors. The sensitivity of convential filter hybridizations using liquid scintillation counting is limited and does not allow the detection of one viral copy per cell unequivocally. The use of autoradiography instead of liquid scintillation counting, however, increases the sensitivity by at least one order magnitude.

A convenient modification of the filter hybridization technique was introduced by *Brandsma* and *Miller* (1980), who adopted bacterial colony hybridization (*Grunstein* and *Hogness* 1975) to eukaryotic cells. A suspension of cells is spotted onto a

nitrocellulose filter. Lysis of the cells and binding of the denatured DNA to the filter is done simply by dipping the filter into an alkaline solution. The obvious advantage is that the technique is very fast and does not require laborious extraction of DNA from many specimens. This makes it particularly useful for diagnostic routine. However, only small amounts of radioactively labeled probe are bound to the filter after hybridization, it is difficult or impossible to discriminate between specific and nonspecific binding. This general problem of filter hybridization can be overcome by the Southern blot hybridization technique.

3.3 Blot Hybridization

DNA fragments generated by site-specific endonucleases and separated on agarose gels can be efficiently transferred to nitrocellulose paper as described by *Southern* (1975). Hybridization with a ^{32}P-labeled probe and subsequent autoradiography thus allows the visualization of specific bands carrying sequences complementary to the probe. Since the binding of the radioactive probe is confined to distinct bands, it is possible to identify even weak signals as specific. Thus, under optimal conditions less than 1 pg SV40 DNA, which corresponds to about 100 000 molecules, may be specifically identified in 20 μg of cellular DNA. If the highest reliability is required, blot hybridization is certainly the method of choice.

3.4 In situ Hybridization

In situ hybridization using labeled DNA or RNA probes followed by autoradiography may be used to search for viral sequences in touch smears or frozen sections (*Gall* and *Pardue* 1971). It offers the advantage that the histology can be evaluated at the same time, allowing the assignment of viral nucleic acids to specific target cells. However, in cases in which only a few grains are observed after autoradiography, it is difficult if not impossible to prove that the hybridization is specific. Present attempts being made in different laboratories to detect sequences of herpes simplex virus (HSV) type 2 in human cancer cells or precancerous lesions are facing this problem (*Eglin* et al. 1981; *Maitland* et al. 1981).

4 Application of Nucleic Acid Hybridization to Diagnostic Problems

4.1 Typing of Herpes Simplex Virus Isolates

Long before the era of monoclonal antibodies, nucleic acid filter hybridization had been used routinely in our institute to type clinical HSV isolates. DNA from cells infected by a clinical isolate and showing a cytopathic effect was bound onto nitrocellulose filters and hybridized with ^{3}H-labeled HSV 1 and HSV 2 DNA in parallel. The amount of labeled probe binding to the filters, in comparison with appropriate control filters containing DNA of HSV 1- or HSV 2-infected cells, allowed typing of the clinical isolate in question (*Schulte-Holthausen* and *Schneweis* 1975).

4.2 Nucleic Acid Hybridization Under Low Stringency as a Tool for the Discovery of New Viruses

As described above, the optimal temperature for hybrid formation is about 25 °C below the T_m. Under these so-called stringent conditions only well-matched duplexes with a high degree of complementarity remain stable. Lowering the hybridization temperature also allows less well matched duplexes to be formed (*Hyman* et al. 1973). Hybridization under conditions of different stringency can be used to study the relationship between families of genes or viruses (*Howley* et al. 1979; *Law* et al. 1979). Figure 1 shows an experiment in which hybridization at different temperatures is used to study the nucleic acid homology of human papillomavirus (HPV) type 1 with HPV 4, 6, and 8, and SV40. The upper panel shows the gel pattern of the DNAs,

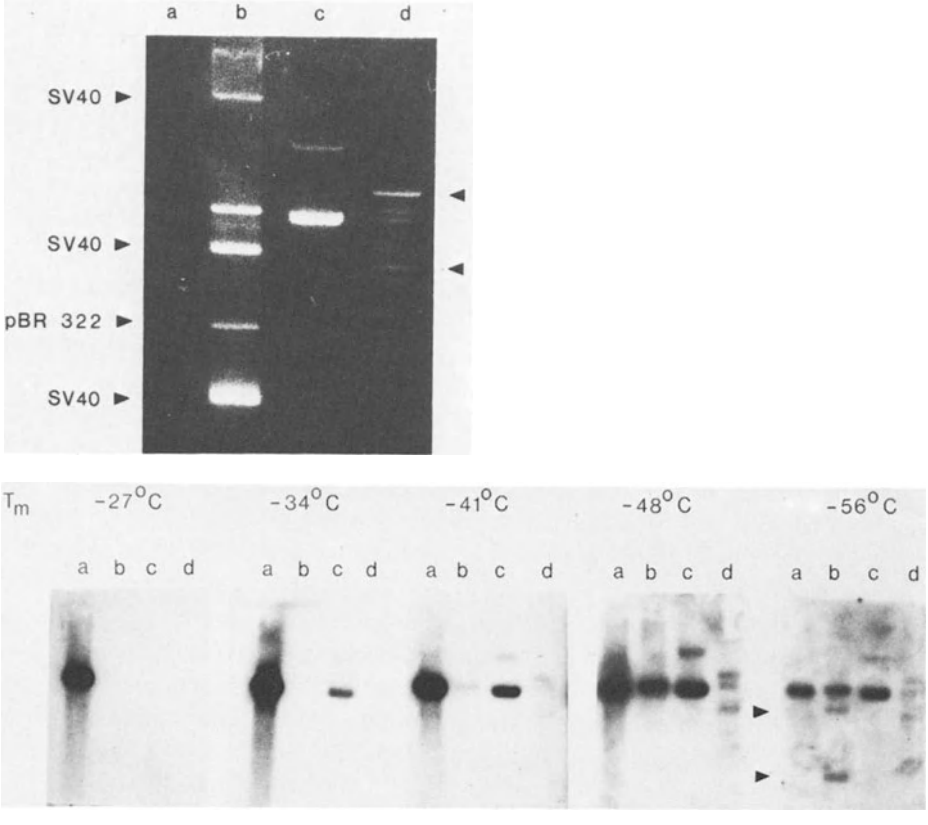

Fig. 1. Cross-hybridization between HPV 4, HPV 6, HPV 8, and SV40 DNA, respectively, and ^{32}P-labeled HPV 1 DNA at different temperatures below the T_m. Identical amounts of DNA were separated on five different 1% agarose gels. The ethidium bromide staining pattern of one of them is shown (*upper panel*). The DNAs were then transferred to nitrocellulose filters that were hybridized to [^{32}P]HPV 1 DNA under different conditions and exposed to X-ray film (*lower panel*). *a*, HPV 1 DNA applied at a low concentration, not visible by ethidium bromide staining; *b*, HPV 4 DNA cloned in pBR322. (The vector was removed by cleavage with *Bam*HI, and SV40 DNA was applied onto the same track (*arrow*). *c*, HPV 6 DNA isolated from a genital wart; *d*, DNA of HPV 8 cloned in two fragments in pBR322 cleaved with *Bam*HI (*arrows*). (The additional bands may be due to incomplete enzyme digestion)

and the lower panel shows the results of the hybridization at five different temperatures. At stringent conditions HPV 1 DNA hybridized exclusively to HPV 1 DNA and not to any other. At 34 °C and 41 °C below the T_m, HPV 1 DNA also hybridized to HPV 6 DNA, but not to HPV 4, HPV 8, and SV40 DNA. At 48 °C below the T_m, HPV 1 DNA also hybridized to HPV 4 and HPV 8 DNA but not to SV40 DNA. At 56 °C below the T_m even the bands of SV40 DNA are visualized with the labeled HPV 1 probe.

This technique of hybridization under conditions of different stringency can be used to search for unknown closely or distantly related viruses. In the case of HPVs this technique is helpful for the identification of new viruses, each type being perhaps associated with specific clinical lesions (*Pfister* 1980). Since HPVs cannot be grown in culture, nucleic acid hybridization is a most important tool for the diagnosis of papillomavirus-associated diseases.

4.3 Sensitive Detection of Epstein-Barr Virus DNA Sequences in Tumor Cell DNA

The consistent presence of Epstein-Barr virus (EBV) DNA and of the EBV-specific nuclear antigen, EBNA, in tumor cells of Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) has strongly linked the virus to these two human malignancies. Regardless of a possible causative role of EBV in the development of these diseases, detection of EBV DNA and EBNA in tumor cells can be helpful in establishing the diagnosis.

The techniques most widely used earlier for the detection of EBV DNA in tumor cells include filter and liquid hybridization (*zur Hausen* et al. 1970; *Nonoyama* et al. 1973; *Lindahl* et al. 1974; *Andersson* et al. 1976; *Bornkamm* et al. 1976). For the analysis of small biopsies and early lesions, which may contain only a small number of tumor cells within a mass normal tissue, these techniques are either not sensitive enough or require too large an amount of DNA.

Here we demonstrate that blot hybridization as described by *Southern* (1975) can be used to detect with high sensitivity the presence of EBV DNA sequences in clinical specimens. The EBV genome is a linear, double-stranded DNA of about 180 kilobase pairs carrying identical repeats of 400 base pairs at both termini (*Given* et al. 1979; *Kintner* and *Sugden* 1979). It is composed of a short unique region of 15 kilobase pairs and a long unique region of 130 kilobase pairs (L_U), separated from each other by an array of 3.2 kilobase-pair repeats in tandem orientation (*Rymo* and *Forsblom* 1978; *Given* and *Kieff* 1979; *Hayward* et al. 1980). The number of internal repeats varies between 6 and 12 among different EBV isolates and is variable even within one DNA population (*Given* and *Kieff* 1978; *Bornkamm* et al. 1980b).

Blot analysis of DNA from tumor tissue with a labeled EBV DNA probe as reported by *Sugden* (1977) turned out to be difficult, since the tumor DNA is often randomly fragmented. Digestion of randomly broken DNA by restriction endonucleases results in a loss of the large fragments, which are replaced by a number of smaller fragments of nonspecific size. Hybridization using the labeled viral genome as a probe thus makes it difficult to detect the pattern of specific viral bands among the diffuse hybridization along the DNA track. This problem can be circumvented by the use of small cloned DNA fragments as hybridization probes instead of the whole viral genome.

We have used the cloned internal EBV repeat, which was kindly provided to us by Dr. S.D. Hayward, to detect EBV sequences in clinical specimens. Since the repeat is represented in the viral genome in several copies, the sensitivity of detection is thus enhanced. Tumor DNAs from four BLs and four NPCs were digested with *PstI*, which cuts twice within the internal EBV repeat. The fragments were separated in 0.8% agarose gels, transferred to nitrocellulose, and hybridized against ^{32}P-labeled internal EBV DNA repeat sequences. The BLs included two EBNA-positive cases observed in Pennsylvania in a cluster of four tumors (*Judson* et al. 1977) and two cases from Germany, one EBNA-positive (*Bornkamm* et al. 1980a) and one EBNA-negative. Two of the NPCs came from Alaska (*Lanier* et al. 1981) and two from Germany. Two different amounts of tumor DNA (4 μg and 1 μg) were applied to the gel.

As shown in Fig. 2, two fragments of 2300 and 900 base pairs are found, representing the two *Pst*I fragments of the internal repeat. Additionally, one or two large fragments of low intensity were visualized, corresponding to the fragments flanking the internal repeat and sharing some of its sequences. In some cases even more bands were detected, either due to heterogeneity of the viral sequences within the tumor or incomplete digestion with the restriction enzyme (Fig. 2, lane 7). As little as 1 μg tumor DNA was sufficient to visualize the viral sequences, and even smaller quantities may be used.

To test the sensitivity of detection we have performed reconstruction experiments adding 0.1, 1 and 5 copies of the internal repeat per cell, as well as 0.1 and 1 copy of the viral genome of the M-ABA (EBV) strain (*Crawford* et al. 1979). As shown in Fig. 3, even 0.1 copy of the repeat per cell could be readily detected. Since a tumor cell normally contains at least ten copies of the viral genome and since one viral genome usually carries 5–12 repeats, it is possible to detect one EBV-carrying tumor cell in about 500–2000 normal cells. However, blot analysis does not allow differentiation between a tumor cell and a normal cell harboring the viral genome.

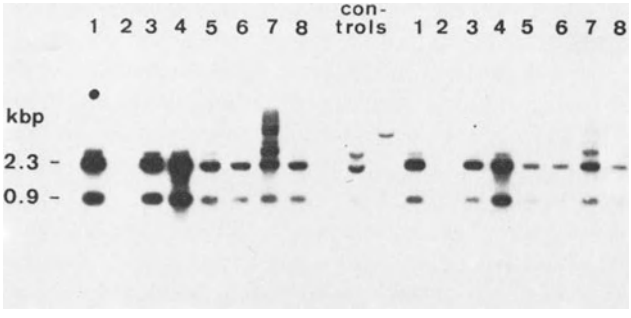

Fig. 2. Blot hybridization of four BLs (*lanes 1–4*) and four NPC biopsies (*lanes 5–8*). *Lane 2* represents an EBV-negative BL. Either 4 μg (*left part*) or 1 μg (*right part*) DNA was digested with *PstI*, the fragments were separated on 0.8% agarose gels, transferred to nitrocellulose, and hybridized with 10^6 cpm/ml nick-translated, cloned B95-8 (EBV) large internal repeat. The controls contained 16 μg DNA of the EBV-negative cell line JM (*Schneider* et al. 1977) either without viral DNA added, with one copy of M-ABA virus DNA per cell, or with one copy of pSL9 DNA per cell (*from left to right*). In the plasmid pSL9 (a gift from S.D. Hayward) the internal repeat is linked to pBR322 sequences giving rise to a larger fragment upon *Pst*I digestion. The blot was exposed for 40 h at −70 °C using an intensifying screen (Agfa-Gevaert MR 600). *Kbp*, kilobase pairs

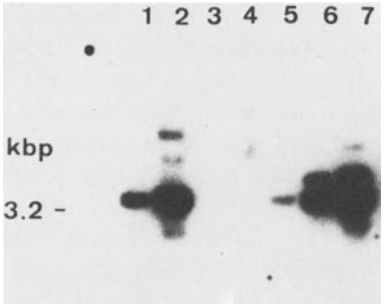

Fig. 3. Reconstruction experiment to test the sensitivity of blot hybridization using the EBV internal repeat. Either 48 pg (*lane 1*) or 480 pg (*lane 2*) *Bam*HI-digested M-ABA virus DNA, or 1.8 pg (*lane 5*), 18 pg (*lane 6*), or 90 pg (*lane 7*) *Bam*HI-digested pSL9 DNA were applied to a 0.5% agarose gel and run for 5 h at 45 V. *Lanes 3* and *4* contained 16 μg JM DNA. After transfer to nitrocellulose the filters were not washed prior to baking at 80 °C for 4 h. Prehybridization and hybridization were carried out in 50% formamide as described (*Bornkamm* et al. 1980b) without dextran sulfate using 2×10^6 cpm/ml of the internal repeat, isolated from a gel and purified from pBR322 sequences prior to nick translation. Because of the high G + C content of the internal repeat, hybridization at 45 °C appears to be important for low background formation. The blots were washed for 5 × 30 min at 52 °C in 0.1 × SSC (0.15 *M* sodium chloride, 0.015 *M* sodium citrate buffer, pH 7.0), 0.1% sodium dodecyl sulfate. The autoradiogram was exposed for 5 days at −70 °C using an intensifying screen. *kbp*, kilobase pairs

The detection of viral sequences by hybridization with the cloned internal repeat is based on the assumption that the internal repeat would invariably be present within the viral genome. Although different virus strains contain different numbers of the internal repeat, there is no indication up to now that EBV strains exist which entirely lack the internal repeat sequences.

Blot hybridization is presently being applied to the analysis of specimens from precancerous and cancerous lesions of the nasopharyngeal mucosa collected in the high risk area for NPC in South China. Patients to be studied are preselected serologically for the presence of serum IgA antibodies against viral capsid antigen (VCA), which are known to develop in NPC patients (*Wara* et al. 1975; *Henle* and *Henle* 1976; *Coates* et al. 1978) before onset of the clinical disease (*Ho* et al. 1978; *Lanier* et al. 1981; *Zeng* et al. 1979, 1981). At least in one case, the diagnosis of a carcinoma in situ was established histologically in a patient reexamined clinically and biopsied again, because EBV DNA was found in the first biopsy (*Desgranges* et al. 1982). Although this and two other reports by *Lanier* et al. (1981) are encouraging, the value of the detection of viral markers (EBV DNA and EBNA) for the early diagnosis of NPC has yet to be established in a careful prospective study, which includes follow-up of all patients over a period of several years.

Because it is impossible and also not desirable to take biopsies from large numbers of persons, *Zeng* et al. (1981) have developed a simple negative pressure suction apparatus to collect exfoliative epithelial cells from the nasopharynx. The cells are collected on a nylon net, examined cytologically, and stained for EBNA on touch

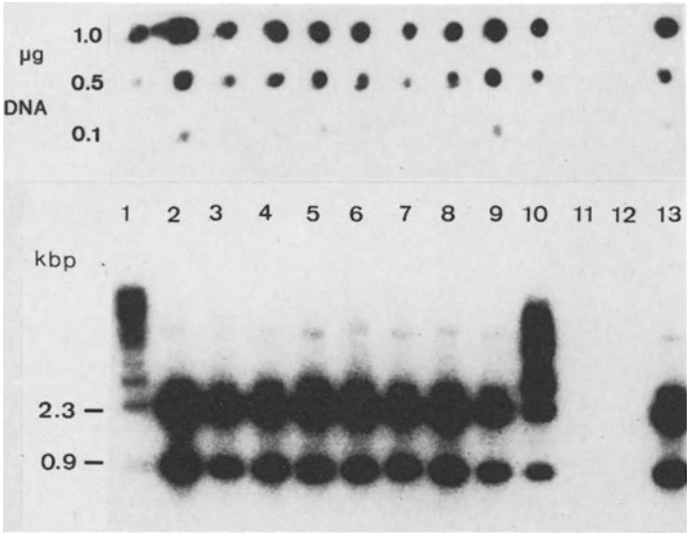

Fig. 4. Blot and spot hybridization of DNA from cells collected by aspiration. In the *upper panel*, DNA of each patient is spotted onto nitrocellulose in the indicated amounts. In the *lower panel*, 16 μg DNA from each patient was digested with *Pst*I, separated on a 0.8% agarose gel, transferred to nitrocellulose filters, and hybridized for 4 days to ^{32}P-labeled purified large internal repeat at 10^6 cpm/ml. The filter was exposed for 20 h at −70 °C using an intensifying screen. *kbp*, kilobase pairs

smears. The amount of DNA extracted from the remaining cells is sufficient to allow blot hybridization.

Figure 4 shows a blot hybridization analysis of specimens collected by aspiration from patients with NPC. In parallel, DNA was spotted onto nitrocellulose in order to evaluate the simpler spot hybridization technique for its potential application in the field. As shown in Fig. 3, of 13 specimens 11 were positive in the blot as well as in the spot hybridization. In all 11 positive cases, clinical, histological, and cytological examination confirmed the diagnosis of NPC, whereas the two negative cases revealed no indication of a tumor by cytological examination and EBNA staining. Suspensions of aspirated cells denatured directly onto nitrocellulose without extraction of DNA have not yet been used in these studies, but will be included in the future. Collection of cells by aspiration thus provides enough material to allow nucleic acid hybridization. It is simple to perform and does not trouble the patient. The only disadvantage is that the rare tumors growing under the mucosa cannot be detected.

Application of nucleic acid hybridization in the field in high risk areas implies several requirements. First, it should be easy to collect specimens, as it now is with the negative pressure suction apparatus. Second, the test should not be too laborious and should permit handling of many specimens at the same time. This might be achieved if the cell suspension, after aspiration, is directly applied of the nitrocellulose without extraction of the DNA. Third, the use of radioactive probes, particularly the use of short-lived isotopes and their detection by autoradiography, is not possible in the field. This latter problem might be overcome in the future by labeling DNA probes with precursors coupled covalently to biotin instead of to [^{32}P]. Biotin-labeled

hybrids may be identified by labeled avidin or anti-avidin antibodies. Avidin or anti-avidin antibodies may be linked to a fluorescent dye or to enzymes, thus eliminating the need to use radioactive isotopes for nucleic acid hybridization. The use of biotin-labeled molecules in nucleic acid hybridization was introduced by *Ward* (personal communication and might provide the basis for a broader application of nucleic acid hybridization to diagnostic problems.

5 Conclusions

Nucleic acid hybridization with labeled viral probes can be used to detect viral sequences in clinical specimens. DNA or RNA is extracted from the specimen and bound to nitrocellulose filters. This is done either directly or after digestion of the DNA with restriction endonucleases and separation of fragments in agarose gels. Nick translation is mostly used to incorporate ^{32}P-labeled precursors into the viral probe. After incubation of the filters with the probe under appropriate conditions, the amount of labeled probe hybridized to the filter is monitored by autoradiography. Because [^{32}P] is short-lived and relatively expensive, nucleic acid hybridization with ^{32}P-labeled probes is not suitable for diagnostic routine and remains restricted to special diagnostic problems. Hybridization under conditions of low stringency may be used to identify sequences of human papillomaviruses in clinical specimens. Since serological tests and an appropriate tissue culture system are not available for human papillomaviruses, nucleic acid hybridization remains the most important diagnostic tool to associate specific clinical lesions with the presence of specific viruses.

Blot hybridization using the ^{32}P-labeled cloned internal repeat DNA of EBV can be used to detect viral sequences in clinical specimens with high sensitivity. As shown by reconstruction experiments, about 1 pg of the internal EBV repeat can be detected. Since the viral genome contains multiple repeats, and a tumor cell generally contains many copies of the viral genome, it is possible to identify one EBV-carrying cell in about 1000 cells. The value of blot and spot hybridization for the detection of precancerous lesions and for the early diagnosis of NPC is presently being investigated in a prospective study in the high risk area for NPC in South China. The problems related to the application of nucleic acid hybridization in the field are discussed.

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The Use of Molecular Hybridization for Demonstration of Adenoviruses in Human Stools

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1 Introduction

Methods for specific detection of nucleic acids have been available for more than two decades. It is therefore surprising that so few efforts have been made to utilize nucleic acid hybridization techniques for identification and typing of microorganisms. Hybridization is a very sensitive method capable of detecting the presence of less than one viral genome per cell and can furthermore discriminate between very closely related nucleic acids. One advantage with hybridization methods, besides the specificity and the sensitivity, is that very small probes representing only a minor fraction of a viral genome can be used for detection. Moreover, by the use of molecular cloning, specific probes can be obtained in large quantities. One problem yet to be solved concerns the label which is used for detection. Probes radiolabeled with [^{32}P] have been most widely used so far. One disadvantage with such probes is that they have a comparatively short half-life and moreover, it is unpleasant to work with large quantities of radioactive phosphorus in a routine diagnostic laboratory. Ideally, a completely different

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type of probe should be developed which is stable and which can be handled without particular safety precautions.

We have recently looked into the possibility of using nucleic acid hybridization methods in clinical virology. In a recent communication (*Stålhandske* and *Pettersson* 1982) we described a simple test system which can be used to detect and type herpes simplex viruses (HSV). In order to evaluate the method further we have used a similar technique for detection of adenoviruses in human stool specimens and compared the results with those obtained by using a four-layer radioimmunoassay (RIA) developed by *Halonen* et al. (1980). We also report preliminary data suggesting that the method can be successfully used for rapid detection of cytomegalovirus (CMV) in urine specimens.

2 Materials and Methods

2.1 Specimens

All stool specimens were obtained from hospitalized children with acute gastroenteritis. A 10% suspension was made in phosphate-buffered saline (pH 7.2) and clarified by low-speed centrifugation.

Nasopharyngeal specimens from hospitalized patients with acute respiratory disease were collected by aspirating the secretion with a disposable mucus extractor through the nostrils from the nasopharynx. The mucus was diluted fivefold with the RIA diluent (20% fetal calf serum, 2% Tween-20, and 0.1% sodium azide in PBS, pH 7.3) and sonicated for 2 min with a Branson Sonifier Cell Disruptor B 15 (Branson Instruments Co., Stanford, Conn., USA) to solubilize the mucus, disrupt the cells, and homogenize the specimens.

Urine specimens were collected from renal allograft recipients.

2.2 Adenovirus Antigen RIA

The RIA used for detection of adenoviruses in stool has been reported in detail elsewhere (*Halonen* et al. 1980). Guinea pig and rabbit immunoglobulins were prepared by immunizing the animals with highly purified hexon antigen of adenovirus type 2. Polystyrene beads were used as the solid phase. The primary catching antibody was the total immunoglobulin (Ig) fraction of guinea pig anti-hexon hyperimmune serum, the secondary antibody was the Ig fraction of rabbit anti-hexon hyperimmune serum, and the indicator antibody was ^{125}I-labeled immunosorbent-purified sheep anti-rabbit IgG antibody. The 10% suspensions of stool specimens in phosphate-buffered saline (PBS) were diluted in RIA buffer to make final dilutions of 1:20, 1:200, 1:2000, 1:20 000, and 1:200 000. The dilution buffer was PBS (pH 7.2) containing 20% fetal calf serum, 2.0% Tween-20, and 0.1% NaN_3.

Diluted stool specimens (200-μl aliquots) were pipetted into disposable polystyrene tubes and a polystyrene bead coated with anti-hexon Ig (0.5 μg per bead) was then added. After 1 h incubation at 37 °C the specimens were aspirated and the beads were washed. Rabbit anti-hexon Ig (200 μl; 16 μg/ml) was then added to each

tube, and the beads were incubated at 37 °C for 1 h. The beads were washed again and 200 µl of the labeled antibody was added to each tube before incubation for another hour at 37 °C. After the washing procedure, the beads were placed in clean tubes and counted in a LKB Wallac 1270 Rackgamma II gamma counter. A buffer reference and reference hexon preparations in concentrations of 100, 10, and 1 ng per ml were included in each assay. The assay was standardized by diluting the iodinated anti-rabbit IgG to a concentration (50 000–130 000 cpm/200 µl) that gave 5000 cpm when a 200-µl volume of the label was incubated with a bead adsorbed with 2 µg of purified rabbit IgG.

A cutoff value of 500 cpm in a dilution of 1:20 was taken, which is approximately twice the mean value for the negative specimens. All specimens giving more than 500 cpm were considered as positive, with the proviso that confirmatory tests (*Halonen* et al. 1980) indicated specific binding. Based on the titration curve of the positive specimens and the curve obtained for the reference hexon preparation, the concentrations in a 1:10 dilution of the stool specimens were calculated.

2.3 Immobilization of Stool and Nasopharyngeal Specimens on Nitrocellulose Filters

A procedure for spot hybridization previously described by *Stålhandske* and *Pettersson* (1982) was essentially followed. All reactions were carried out in Eppendorf centrifuge tubes. Undiluted suspension (100-µl aliquots) was treated with proteinase K at a concentration of 50 µg/ml for 1 h at 37 °C. The sample was then diluted with an equal volume of distilled water and 200 µl of phenol was added. The water phase was extracted once before addition of sodium acetate to a final concentration of 0.3 *M*. Three volumes of ethanol were added and the resulting precipitate was collected by centrifugation. The pellet was dried under vacuum, then dissolved in 200 µl 0.3 *M* NaOH and boiled for 5 min. Tris-HCl buffer, pH 7.5 was added from a 1-*M* stock solution to make a final concentration of 0.1 *M*, and the sample was finally neutralized with HCl after being placed in an ice bath. The sample was then mixed with half a volume of 20 × SSC (SSC, 0.15 *M* NaCl, 0.015 *M* sodium citrate). Aliquots (50 µl) were applied to a nitrocellulose filter under mild negative pressure. The filter was rinsed in 6 × SSC after application of the sample and subsequently dried at 37 °C and baked under vacuum at 80 °C for 2 h.

2.4 Immobilization of Urine Specimens on Nitrocellulose Filters

Aliquots (5 ml) of urine were centrifuged in an SW50 rotor at 25 000 rpm for 60 min. The pellet was dissolved in 200 µl 0.01 *M* Tris-HCl, pH 7.4, 0.001 *M* EDTA and treated for 10 min at room temperature with 100 µl 3% Sarcosyl in 0.075 *M* Tris-HCl, 0.025 *M* EDTA, pH 9.0. Then 0.5% pronase (75 µl; autodigested at 37 °C for 2 h) was added and incubation continued at 37 °C for 2 h. After extracting twice with phenol:chloroform (1:1) and three times with ether, 1/10 volume of 3 *M* sodium acetate was added and the sample precipitated with two volumes of ethanol. The DNA was finally dissolved and fixed to a nitrocellulose filter as described above.

2.5 Hybridization

The filters were presoaked for 2 h at 65 °C in a solution which contained 6 × SSC, 5 × Denhardt's solution (*Denhardt* 1966), and 50 μg/ml salmon sperm DNA. Then the probe, consisting of 10 000 cpm/sample adenovirus type 2 DNA labeled in vitro with [^{32}P] according to *Rigby* et al. (1977), was added together with sodium dodecyl sulfate (SDS) to a final concentration of 0.5%. The specific activity of the probe was about 1×10^7 cpm/μg DNA. The filters were washed in 2 × SSC, 0.5% SDS after hybridization at 65 °C for 6 h. Hybridization was revealed by autoradiography using intensifying screens.

For detection of CMV in urine, a hybridization probe consisting of a cloned CMV fragment inserted into the plasmid pBR322 (*Gadler* et al., unpublished data) was used. The fragment was labeled in vitro to a specific activity of approximately 2×10^6 cpm/μg.

3 Results

3.1 A Simple Assay for Detection of Viral Nucleic Acids

We have recently used a model system consisting of cells infected with HSV types 1 and 2 in order to design a nucleic acid hybridization method which could be used for diagnostic purposes. The results showed that the method is sensitive and can be used for detection of HSV. Moreover, by the use of probes consisting of cloned restriction enzyme fragments it was possible to discriminate between the two HSV serotypes (*Stålhandske* and *Pettersson* 1982).

3.2 Detection of Adenoviruses in Human Stool Specimens

In order to test the sensitivity of the method and to evaluate its general applicability we decided to analyze stool specimens from patients with gastroenteritis. The speci-

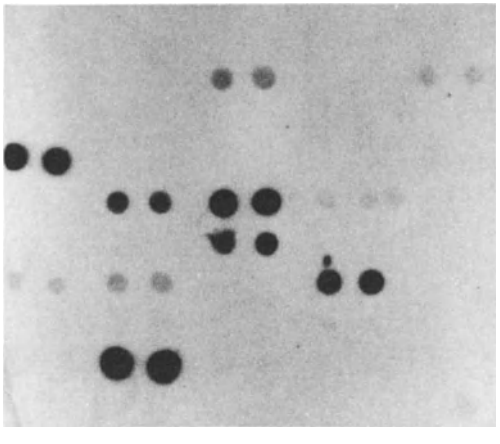

Fig. 1. Analysis of stool specimens by spot hybridization. Duplicate samples were immobilized on a nitrocellulose membrane. ^{32}P-labeled adenovirus type 2 DNA was used as a probe for hybridization. Samples which range from negative to +++ are shown. The exposure time was 2.5 h

Table 1. A comparison between the spot hybridization method and RIA

Amount of hexon antigen according to RIA (μg/ml)	Hybridization[a]
200	+++
5	++
1	+
0.5	+
<0.02[b]	0
<0.02	0
<0.02	0
<0.02	0

[a] Hybridization was determined semiquantitatively according to an arbitrary scale in which no hybridization is designated "0" and a homogeneously dark spot "+++" after 24-h exposure; [b] Specimens containing <0.02 μg/ml hexon antigen are considered to be negative

Table 2. A comparison between RIA and the spot hybridization method

	Results obtained by spot hybridization				
	Negative	(+)	+	++	+++[b]
25 negative specimens (RIA)	24	1	0	0	0
24 positive specimens (RIA)	3[a]	1	8	8	4

[a] 0.02–0.2 μl/ml according to RIA; [b] Hybridization was determined semiquantitatively according to an arbitrary scale in which no hybridization is designated "Negative" and a homogeneously dark spot "+++" after 24 h exposure

mens were analyzed both by a recently developed radioimmunoassay and by the spot hybridization method described in Sect. 2, using ^{32}P-labeled adenovirus type 2 DNA as a probe (Fig. 1). Tests using ^{32}P-labeled bovine papillomavirus DNA were included as negative controls. In the first experiment four stool specimens which were scored as positive by RIA, as well as four negative samples, were included. A very good correlation between the results obtained by the two methods was obtained in this study (Table 1).

In order to further evaluate the method, 49 blind-coded samples were analyzed by the two procedures. The results from this analysis are summarized in Table 2. All samples which were found to be negative by RIA were, with one exception, also found to be negative when analyzed by the spot hybridization assay. Control experiments using an unrelated probe showed that the observed hybridization was specific in each case. Among those which were scored positive there was in most cases a good correlation between results obtained by the two methods, i.e., samples which contained high concentrations of antigen were usually scored as +++ in the hybridization assay.

There were, however, a few exceptions to this rule. Three samples which were positive in the RIA were scored as negative by the hybridization assay. However, the antigen concentrations in all these cases were estimated to be low, 0.02–0.2 μg/ml. Some quantitative differences were also striking; for instance one specimen which was estimated to contain only 0.7 μg/ml antigen was scored as +++ in the hybridization assay, and one specimen containing 20 μg/ml of antigen was only weakly positive by hybridization.

3.3 Analysis of Nasopharyngeal Specimens

A set of ten specimens which included both positive and negative samples as judged by RIA were immobilized on nitrocellulose and analyzed by hybridization using ^{32}P-labeled adenovirus type 2 DNA or bovine papillomavirus DNA as probes. All specimens gave positive results irrespective of which probe was used, which suggests that the results are nonspecific.

3.4 Detection of CMV in Urine Specimens

A model system for detection of CMV in urine was designed. An extracellular preparation of CMV was diluted in urine from a healthy person lacking antibodies against CMV. The samples were immobilized on nitrocellulose as described in Sect. 2 and hybridized with a cloned ^{32}P-labeled fragment of CMV DNA. The results from the reconstruction experiment, which are shown in Fig. 2, indicate that a concentration of approximately 2.5×10^4 fluorescent focus units/ml (FFU) of urine is detectable by the hybridization method. Urine specimens collected from patients with suspected CMV infections were tested according to a similar protocol and one example is shown in Fig. 2 (*f*). When analyzed by conventional isolation methods, 21 days were needed in order to obtain a positive result for the same specimen.

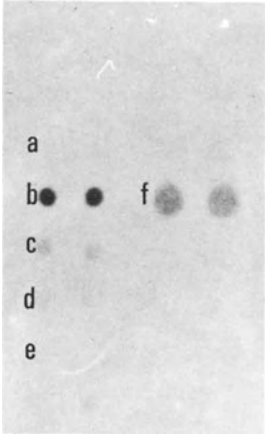

Fig. 2. Detection of CMV in urine specimens. CMV diluted in urine from a normal person (*a–e*) and a urine specimen from a renal allograft recipient (*f*) were analyzed. Specimen *f* was also positive as revealed by virus isolation. Duplicate samples were analyzed in each case. Autoradiography was performed overnight. *a*, urine without CMV; *b*, 10^6 FFU/ml; *c*, 2.4×10^5 FFU/ml; *d*, 6.3×10^4 FFU/ml; *e*, 1.6×10^4 FFU/ml

4 Discussion

The present study shows that nucleic acid hybridization methods can be used in viral diagnosis. The main advantage of this method besides its high sensitivity is that it can discriminate between very closely related viruses. Highly specific probes can be constructed either by cloning small restriction fragments or by chemical synthesis of DNA segments wich should then allow specific identification of almost any virus. The procedure which is described in the present communication has not been optimized, and the sensitivity could probably be increased 10- to 100-fold, simply by increasing the specific activity of the probe. With the present version of the method, results can be obtained within 20–30 h. Several of the steps involved could, however, be shortened and we anticipate that when optimized, the assay should provide results within a few hours.

A few problems have been encountered when applying the method to clinical specimens. An analysis of nasopharyngeal specimens indicates that nonspecific hybridization occurs, since probes consisting of both adenovirus DNA sequences and bovine papillomavirus DNA sequences yielded positive results. The cause of this nonspecific binding is unknown but could be related to the presence of components in the specimens which nonspecifically adsorb DNA to the nitrocellulose filter. We consider this to be a technical problem, however, which most likely could be solved by pretreating the samples in a different way before immobilization. When comparing the spot hybridization assay with the RIA used for detection of adenovirus antigens, it became clear that there was not an absolute correlation between results obtained by the two procedures. Results which were positive with one method were in nearly all cases positive in the other, but in some cases there were quantitative differences. These differences could be related to the fact that the two methods assay different components. It is, for instance, conceivable that samples which contain high concentrations of DNA and little protein have been degraded due to proteolytic activity in the specimen. Another important factor to take into account is the nature of the probe used in the study of stool specimens. Although not yet typed, we anticipate that most of the positive specimens contain adenoviruses belonging to the recently described subgroup F. The sequence homology between adenovirus type 2 and the subgroup F adenoviruses is expected to be rather limited.

We believe that molecular hybridization could be developed into a powerful diagnostic tool. The hybridization procedures are comparatively easy to carry out and automatic methods could probably be designed. The future of the technique will, however, to some extent depend on whether labels other than radioactivity can be developed for detection. Due to the rapid progress in the field of nucleic acid chemistry it is reasonable to anticipate that alternative probes will be developed in the near future.

5 Conclusions

A simple method has been developed for identification of viral nucleic acids in clinical specimens. The total DNA of the specimen is immobilized on nitrocellulose filters and the presence of viral nucleic acid sequences is revealed by molecular hybridization

using either purified viral DNA or cloned restriction fragments of viral DNA as a probe. The method has been evaluated using cells infected with HSV types 1 and 2 as a model, and the results demonstrate that the method is sensitive and capable of discriminating between the two herpesvirus serotypes.

This method has also been applied to the identification of adenoviruses in specimens consisting of human stool. The specimens were immobilized on nitrocellulose filters following a simple extraction procedure, and the presence of viral DNA was demonstrated by hybridization. This method was compared with the four-layer RIA recently developed by *Halonen* et al. (1980), and the results showed that the method is comparable in sensitivity and as specific as RIA. In most cases a good correlation was found between the amount of antigen detected by RIA and the amount of DNA detected by hybridization.

Finally, the method was used for identification of CMV in urine. Urine specimens from patients suspected of having CMV infections were concentrated by centrifugation before immobilization on nitrocellulose. Positive results were obtained within a total analysis time of 48 h.

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Nucleic Acid Sandwich Hybridization in Adenovirus Diagnosis

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1 Introduction

The usefulness of viral diagnosis is critically dependent on the rapidity by which it is able to detect and identify virus taken from the site of infection. The methods suitable for rapid diagnosis so far available are mostly based on immunological detection of the virus and its antigenic components (for a review, see *Halonen* et al., this volume). An alternative to these techniques worth considering is provided by nucleic acid hybridization techniques to detect virus-specific nucleic acid sequences in the sample (for principles and applications see reviews by *Bornkamm* et al. and *Petterson* et al., this volume).

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The sensitivity and rapidity by which the virus can be identified by the two techniques are similar. Nucleic acid hybridization offers, however, some specific advantages.

1. It has a higher specificity than conventional immunological methods and it is not hampered by cross-reactions. Moreover, the specificity can in most cases be adjusted to the desired level by correct selection of reagents and reaction conditions (*Stalhandske* and *Pettersson* 1982).

2. Nucleic acid hybridization is a useful tool in distinguishing between virulent and avirulent strains of a single organism if the gene responsible for the virulence factor is carried on a plasmid (*Ben Gurion* and *Shafferman* 1981; *Gemski* et al. 1980). Using nucleic acid hybridization, *Moseley* et al. (1980) have diagnosed diarrhea caused by enterotoxigenic *Escherichia coli* by showing the presence of the virulence gene in stool samples.

3. There are infections such as those caused by viroids in which the infectious agent can only be demonstrated by the presence of its nucleic acid. Thus, potato spindle tuber viroid has been demonstrated in infected potatoes by nucleic acid hybridization (*Owens* and *Diener* 1981).

Hybridization methods are now becoming more easily available even for routine diagnosis because reagents (i.e., probes) can be made without difficulty by recombinant DNA techniques. As soon as cloned genomes, or fragments thereof, of different microorganisms become available, they can be propagated indefinitely at low cost.

We are developing a hybridization method that could be used for screening large numbers of samples and would be a general method applicable for the detection of DNA and RNA viruses, as well as bacteria. To be suitable as a routine diagnostic method, a prerequisite is that the procedure should require a minimum number of manipulations. We think that the sandwich hybridization technique originally described by *Dunn* and *Hassell* (1977) could be modified to fulfill these criteria. The method was designed as a two-step reaction for mapping adenovirus transcripts from an infected cell. Our modified procedure also detects double-stranded DNA in a one-step reaction and it can successfully be applied to microbial diagnosis.

The sandwich hybridization method is based on the use of two separate nucleic acid reagents derived from the organism to be identified (*Dunn* and *Sambrook* 1980). One of them is immobilized on a solid phase, a nitrocellulose filter, whereas the other, which is radioactively labeled, functions as a soluble probe that finally labels the hybrid in order to enable its detection. Without the correct nucleic acid molecules present in the sample, the probe does not bind to the filter. A filter-bound hybrid with the radioactive probe is formed, however, if homologous DNA is present in the sample solution. The principle of the sandwich hybridization is shown in Fig. 1B.

2 Materials and Methods

2.1 Reagents

2.1.1 Nucleic Acids Used as Probes and on Filters

All nucleic acid reagents were prepared using recombinant DNA techniques. Two separate fragments derived from the genomes of the organisms to be identified were

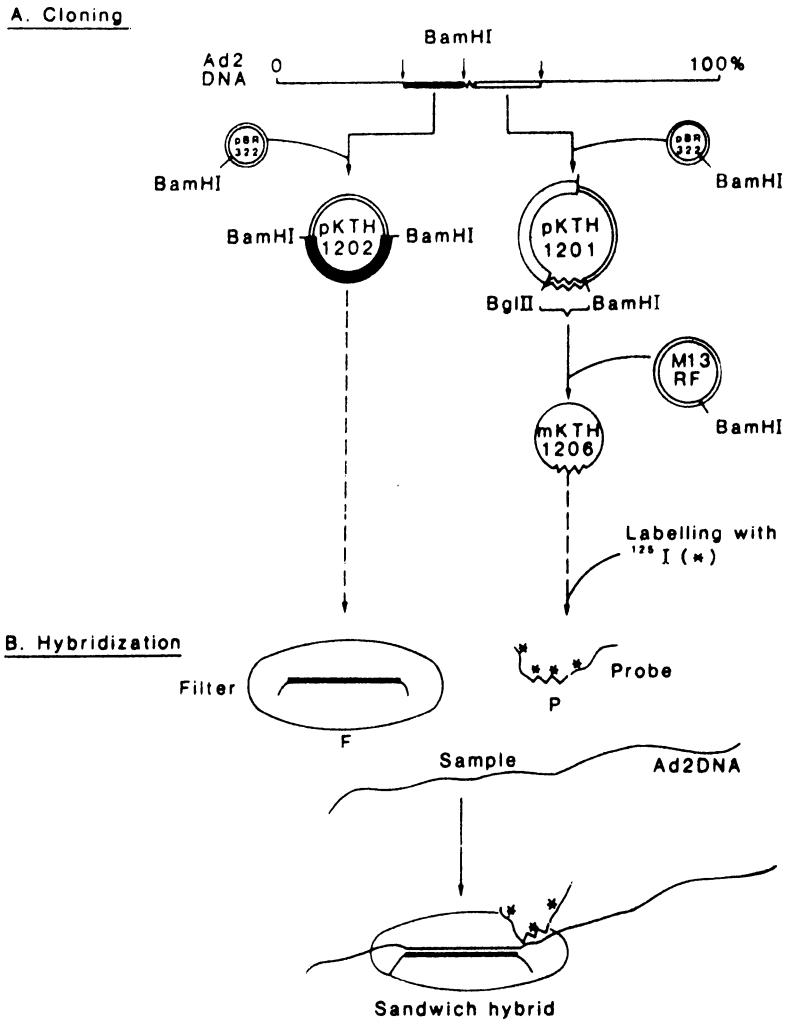

Fig. 1A. Cloning procedures for producing the two separate nucleic acid reagents F-DNA and P-DNA used for detecting Ad2 DNA. B) Principle of the sandwich hybridization test

cloned into two separate vector molecules, the plasmid pBR322 (*Bolivar* et al. 1977; *Clewell* and *Helinski* 1969) and the single-stranded DNA phage M13 (*Heidecker* et al. 1980; *Messing* et al. 1981). The principles of reagent preparation using adenovirus type 2 (Ad2) as a model are described in Fig. 1A. One of the recombinant DNA molecules was immobilized on nitrocellulose filters (F-DNA), and the other was labeled with [^{125}I] (*Commerford* 1971) and used as probe (P-DNA). Similar reagents were prepared from adenovirus type 3 (Ad3) DNA and from E. coli. For the latter, a plasmid containing the *ompA* gene (*Henning* et al. 1979) was used as source of DNA for subclonings.

Reagents for identification of Semliki Forest virus (SFV) were restriction fragments derived from a recombinant plasmid containing a cDNA copy of part of the viral genome (Recombinant DNA Laboratory, University of Helsinki). The fragments

were separated by electrophoresis on agarose gels and eluted (*Wieslander* 1979). P-DNA was labeled by nick translation using [^{125}I]dCTP as substrate (*Rigby* et al. 1977).

The recombinant DNA clonings were performed according to the instructions of the Finnish National Board of Health Committee for Recombinant DNA Research.

2.1.2 Sample Nucleic Acids

Ad2 DNA was obtained through Bethesda Research Laboratories, Inc. Ad3 DNA was obtained by phenol extraction of purified virus grown in A-549 cells (*Pettersson* and *Sambrook* 1973). Semliki Forest virus, a prototype strain, was propagated in BHK21 cells. Infected cells were collected into 2% sodium dodecyl sulfate (SDS), viscosity due to chromosomal DNA was reduced by mechanical shearing, and the samples were finally denatured at 100 °C for 5 min.

2.2 Hybridization

Sandwich hybridization was carried out as depicted in Table 1. Controls I and III represent the nonspecific radioactive background remaining on the filters after washing,

Table 1. Nucleic acid sandwich hybridization; optimal conditions and controls[a]

Reagents and treatments (400-μl reaction)	Test	Controls		
		I	II	III
Salt and background-reducing reagents	+	+	+	+
4 × SSC				
0.02% polyvinylpyrrolidone and ficoll				
0.25% SDS				
200 μg/ml sheared and denatured herring sperm DNA				
Nucleic acid reagents				
Filters[b] (F-DNA)				
Ad-specific (0.4 μg pKTH 1202 DNA)	+	–	+	–
Control (0.4 μg calf thymus DNA)	–	+	–	+
Probe (P-DNA)				
mKTH 1206 DNA labeled with [^{125}I]	+	+	+	+
sp. act., 10^7–10^8 cpm/μg; 200 000 cpm/reaction				
Sample (denatured in 1% SDS at 100 °C, 5 min)	+	+	–	–
Incubation	+	+	+	+
65 °C overnight (17–20 h)				
Washing	+	+	+	+
37 °C for 2 h in 0.1 × SSC, 0.2% SDS with stirring				
Detection	+	+	+	+
Counting for [^{125}I]				

SSC, 0.15 *M* sodium chloride, 0.015 *M* sodium citrate, pH 7.0

[a]Exemplified for Ad2 DNA test; [b]10-mm-diameter nitrocellulose filters to which the DNA is fixed

whereas control II characterizes the specific reagent background (i.e., binding of P-DNA to F-DNA without sample). Results are usually expressed as specific counts, per minute after subtracting control II from the radioactivity hybridized to the test filter. In some experiments (indicated in the table indexes), hybridization was carried out in 50% formamide at 37 °C, but otherwise as described in Table 1.

2.3 Clinical Specimens

Nasopharyngeal mucus specimens were collected from children with acute respiratory infection. The specimens were diluted fivefold with radioimmunoassay (RIA) dilution buffer (20% fetal calf serum, 2% Tween 20, 0.02% merthiolate in phosphate buffered saline, pH 7.3 [PBS]) and sonicated. Before testing in the sandwich hybridization assay they were further diluted with an equal volume of 2% SDS in distilled water, treated with proteinase K (1 mg/ml, 37 °C, 1 h), denatured at 100 °C for 5 min followed by quick cooling and addition to the test.

2.4 Radioimmunoassay

The hexon antigen content of the nasopharyngeal specimens was determined as described previously (*Halonen* et al. 1980; *Sarkkinen* et al. 1981).

2.5 Virus Isolation

Virus in the clinical specimens was pelleted through a 0.5-ml cushion of 30% sucrose in PBS in an SW 50.1 rotor for 2 h at 40 000 rpm. The pellets were resuspended in PBS and inoculated into cultures of primary human amnion cells. Virus growth was detected by cytopathic effect and the virus typed by the hemagglutination inhibition test.

3 Results and Discussion

3.1 Sandwich Hybridization

Reaction conditions of the sandwich hybridization test with regard to salts, temperature, and background-reducing reagents were optimized using Ad2 DNA as the sample (Table 1) (*Ranki* et al., in preparation). Hybridization at 65 °C without formamide was three times as efficient as hybridization in the presence of 50% formamide at 37 °C after an equal incubation period (17 h). Dextran sulfate did not increase hybridization efficiency as compared with its effect on direct hybridization using a double-stranded probe (*Wahl* et al. 1980). On the contrary, it had a negative effect by increasing the background. The Denhardt reagents (*Denhardt* 1966) were always added to the hybridization mixture, but without bovine serum albumin. We found that carrier protein was not necessary, but the presence of nonspecific denatured carrier DNA, both

during hybridization and presoaking of the filters, was important in reducing the background of the assay to a reproducibly low level.

It was crucial to optimize the concentrations of the DNA reagents to levels at which they would not limit the efficiency of hybridization. This was easily achieved with F-DNA because the binding capacity of the filter is very high (*Kafatos* et al. 1979). The reaction is also dependent on P-DNA so that the efficiency of labeling the filter-bound hybrid increases with increasing probe concentrations up to around a 100-fold probe excess as compared with the sample concentration. It is, however, not practical to carry out the reactions with very high amounts of radioactivity. Moreover, the use of optimal probe concentrations is only important at the lowest sample levels, at which the sensitivity of the assay becomes critical. In practice we have carried out the hybridizations using 200 000 cpm P-DNA per assay, which gives at least a 100-fold concentration excess with samples containing less than 0.5 ng DNA.

The reaction kinetics and sensitivity of the sandwich hybridization are shown in Table 2. The results demonstrate that an overnight incubation is sufficient for detection of 0.2 ng of adenovirus DNA, which corresponds to 5×10^6 molecules. Longer incubation periods increase the background to an undesirable level in the Ad2 test, in which we always have a certain amount of reagent background (Table 1, control II).

3.2 Clinical Samples

3.2.1 Selection of Reagents

Adenoviruses are divided into six groups on the basis of DNA homology (*Sambrook* et al. 1980). According to determinations by *Green* et al. (1979) the overall sequence homology of representative viruses within a serogroup is 80%–90%, whereas that between viruses of different serogroups is only 10%–15%. We tested whether the cloned fragments of Ad2 DNA would show higher cross-reactivity to Ad3, knowing that the clone pKTH 1201 (Fig. 1) includes the gene for the group-specific hexon antigen. Table 3 confirms the low cross-reactivity (20%–30%) between Ad2 and Ad3. It also shows that in spite of common antigenic determinants, the nucleotide homology can be low (hybridization of pKTH 1201 DNA to Ad3 DNA). We therefore prepared a

Table 2. Sensitivity of the sandwich hybridization test[a]

Sample DNA (ng)	P-DNA (cpm/μg $\times 10^{-7}$)	Hybridization time (h)				
		6	10	20	30	46
50	2	968 (9.4)	1760 (13)	3167 (19)	3452 (12)	
5	2	189 (2.6)	526 (4.6)	933 (6.4)	914 (3.9)	1008 (6.6)
0.5	7	34 (1.2)	50 (1.6)	208 (2.7)		233 (2.1)
0.2	8			198[b] (1.7)		

[a]Specific cpm on pKTH 1202 filters. In brackets is given the ratio of test: control II (Table 1); [b]Mean of four parallel determinations with a range of 180–205. Mean control (II) was 118 cpm and the cut-off line below which 97.5% of the negatives distributed was 172 cpm

Table 3. Cross-hybridization between Ad2 and Ad3 DNA

Probe[a]	Hybridization[b] to filter containing	
	Ad2 DNA	Ad3 DNA
Ad2 DNA	46 500	8 670
pKTH 1201	41 000	3 550
pKTH 1202	39 500	9 180
Ad3 DNA	8 220	35 100

[a]DNA preparations were labeled by nick translation using [^{125}I]dCTP as substrate to a sp. act. of approx. 4 × 10^7 cpm/μg DNA. 200 000 cpm were added to each hybridization; [b]Cpm on filter containing 0.8 μg of the indicated DNA. Hybridization was for 24 h at 37 °C in the presence of 50% formamide

separate set of reagents for detection of each of the serogroups considered to be clinically important.

Because most of the adenoviruses causing respiratory infections among children belong to serogroups C and B, we tested whether reagents prepared from Ad2 DNA (member of the group C) and from Ad3 DNA (member of the group B) were sufficient for detection of different members within the groups (Table 4). Extracts of cells infected with the indicated adenoviruses were used as samples. The results show that viruses within one serogroup can be detected with one set of reagents, the efficiency of the test being probably somewhat lower for the nonidentical serotypes. The quantitative results of the hybridization should, however, be considered with caution due to the possible differences in the amount of virus produced by different virus types.

Table 4. Detection of adenoviruses from serogroups B and C by the sandwich hybridization assay

Virus	Reagents[a]	Number of infected cells (×10^{-3})[b]						DNA (ng)
		50	16	12	4	3	1	1
Ad1	2		1270		295		131	
Ad2	2		6911		4243		1060	250
Ad5	2		3692		1391		356	
Mock	2	8						
Ad3	3	873		363		147		257
Ad7	3	476		143				
Mock	3	2						

[a] 2, indicates that the test was for serogroup C adenoviruses using pKTH 1202 as F-DNA and mKTH 1206 as P-DNA (7 × 10^7 cmp/μg). 3, indicates the serogroup B test using reagents prepared from Ad3 DNA; [b] A-549 cells, infected at a multiplicity of infection of 5–10 plaque-forming units/cell, were collected 24 h post infection into 1% SDS. The extract was sheared mechanically, boiled, and subjected to testing. Results are given as specific cpm

3.2.2 Clinical Specimens and Testing Conditions

In order to evaluate the applicability of the sandwich hybridization assay to viral diagnosis, nasopharyngeal specimens were selected to be tested for the presence of adenovirus. The specimens were taken from children with acute respiratory infections and had been previously tested for the presence of adenovirus hexon antigen by RIA. The coded specimens were subjected to the sandwich hybridization test as well as to virus isolation. Nucleic acid reagents specific for serogroups B (Ad3) or C (Ad2) were used in the hybridization assay.

The samples were supplied in a condition optimal for antigen detection, but not necessarily for nucleic acid determination. The effect of their content (mucus and RIA dilution buffer containing a high concentration of protein) on the hybridization efficiency was studied. Table 5 shows that only 120 μl of the clinical specimen could be added to the hybridization assay of 400 μl without strong inhibition of the reaction. If the samples had been prepared specifically for the hybridization assay, a larger volume of the sample could probably have been tested, because mucus itself did not show an inhibitory effect additional to that observed with the buffer alone.

3.2.3 Results of the Sandwich Hybridization Test

The hybridization results (Table 6) indicated that five samples out of the ten tested were positive when assayed with the Ad2 reagents (ratio of cpm to control II >2). None was positive with the Ad3 reagents. The correlation of the results obtained by RIA and sandwich hybridization was 100%. The virus isolation experiment served as an independent control for both of the rapid assays. It confirmed that all the samples which were scored positive in the hybridization assay with the Ad2 reagents in fact contained a group C adenovirus, either Ad1 or Ad2. It also showed that none of the rapid assays could compete in sensitivity with virus isolation: two additional adenovirus positive samples were detected, both being of type 1. The difference in sensitivity

Table 5. Inhibition of nucleic acid hybridization by buffer components included in the nasopharyngeal specimens

Quantity (μl)	Addition to the reaction[a]	
	Negative specimen	RIA dilution buffer
0	3144	3144
120	2821	3003
150	1974	2088
180	2394	740
210	563	360
240	412	

[a] The given amounts of an adenovirus-free nasopharyngeal specimen taken up in RIA dilution buffer or the buffer alone were treated with 1 mg/ml proteinase K at 37 °C for 1 h before addition to the 400-μl (final volume) hybridization mixture. Each reaction contained 20 ng Ad2 DNA. The corresponding controls without added DNA (control II) are subtracted

Table 6. Nucleic acid sandwich hybridization test of nasopharyngeal specimens from children

Sample no.	Sandwich hybridization[a]		RIA (Hexon)	Virus isolation
	Ad2	Ad3		
10	1901 (11.4)	68	+	Ad1
8	1514 (9.3)	30	+	Ad1
3	1492 (9.2)	0	+	Ad2
6	336 (2.8)	31	+	Ad2
5	204 (2.1)	93	+	Ad2
1	67 (1.4)	31	–	–
2	109 (1.6)	35	–	–
4	98 (1.5)	104	–	–
7	89 (1.5)	95	–	Ad1
9	89 (1.5)	40	–	Ad1
50 ng Ad2 DNA	1411 (8.7)			
10 ng Ad2 DNA	719 (5)			
2 ng Ad2 DNA	210 (2.1)			
50 ng Ad3 DNA		1712 (10.1)		
10 ng Ad3 DNA		856 (5.5)		
2 ng Ad3 DNA		285 (2.5)		

[a] Specific cpm hybridized using Ad2 or Ad3 reagents as indicated. Hybridization was for 16 h at 65 °C with probes having a sp. act. of 7 × 10^6 cpm/μg DNA. Values in brackets indicate ratio of test: control II (Table 1)

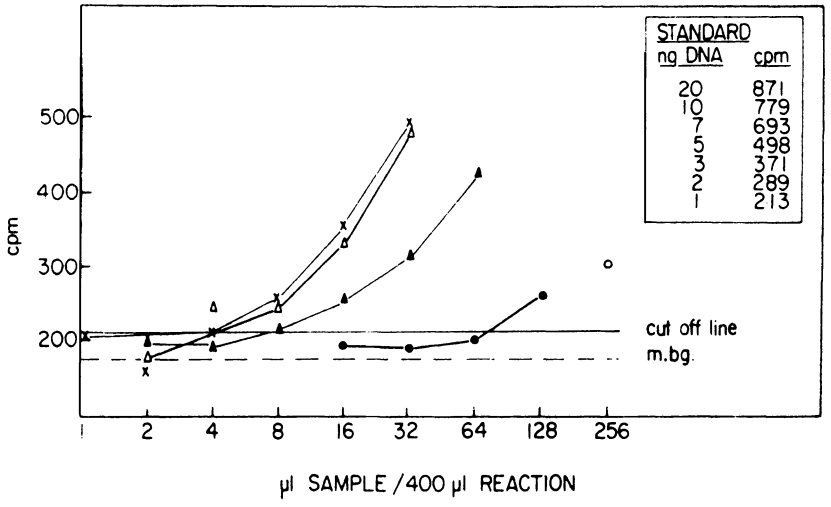

Fig. 2. Nucleic acid sandwich hybridization test of clinical nasopharyngeal specimens. Prior to testing, the samples (in RIA dilution buffer) were diluted with an equal volume of 2% SDS in distilled water and treated with proteinase K. Cpm on pKTH 1202 filters are shown. The mean background (*m.bg.*) as measured by six parallel reactions with no sample DNA is shown as well as the cut-off line indicating the level below which 97.5% of the negatives distributed. x——x, sample no. 10; △——△, no. 3; ▲——▲, no. 8; ●——●, no. 6; ○, no. 5

was not unexpected because even a few infectious virus particles in the sample are sufficient to allow detection by isolation. It is, however, important to remember that the hybridization assay as well as the RIA gave the result after an overnight incubation, whereas up to 3 weeks were required before all of the isolations became positive.

Titrations of the samples positive in the RIA and nucleic acid hybridization tests were carried out to compare the sensitivities of the two methods. The results of the sandwich hybridization (Fig. 2) clearly confirm that five of the samples were positive compared with an adequate series of background tests. The results also show that dilutions greater than 100-fold were no longer positive in the nucleic acid test as opposed to the RIA, in which some of the samples could be diluted up to 100 000-fold. This indicates that demonstration of hexon antigen is a particularly sensitive way to diagnose adenovirus, at least from nasopharyngeal secretions. Nevertheless, all samples positive for hexon antigen were also positive in the nucleic acid test. According to an additional series of 50 nasopharyngeal specimens, the sensitivities of the sandwich hybridization and of the RIA are similar in detecting adenovirus-positive samples. In this series we also detected infections caused by serogroup B viruses (*Virtanen* et al. 1982).

3.3 Sandwich Hybridization as a General Method in Microbial Diagnosis

Table 7 shows the results of experiments carried out to detect two additional types of microbes by the sandwich hybridization assay: Semliki Forest virus, as an example of an RNA virus, and *E. coli* were chosen as models. The results indicate that RNA can also form a stable sandwich hybrid, provided ribonuclease activity is prevented by, e.g., SDS. It also shows that an organism having a 100-times larger genome than adenovirus can be detected by sandwich hybridization using an approximately 2500-base-pair fragment.

Table 7. Applicability of the sandwich hybridization for detection of RNA virus and bacteria

Sample	Hybridization	
	ompA reagents	SFV reagents
E. coli HB101 DNA (1.46 μg)	666[a]	
SFV-infected cells (5×10^5)		2680[b]

[a] Specific cpm hybridized to the *E. coli*-specific filter after hybridization for 19 h at 65 °C with 150 000 cpm of P-DNA (sp. act. 5×10^7 cpm/μg); [b] Specific cpm hybridized to the SFV-specific filter after hybridization for 20 h at 37 °C in the presence of 50% formamide. 200 000 cpm of P-DNA (sp. act. 9×10^7 cpm/μg)

4 Conclusions

A nucleic acid sandwich hybridization test has been developed for screening large numbers of samples in a rapid and sensitive way. It is a general method applicable for detection of DNA and/or RNA. The nucleic acid reagents required in the test are produced by recombinant DNA techniques by cloning into two separate vectors, which enabled the use of the entire recombinant DNA molecules in the test.

The sandwich hybridization technique offers some specific advantages as compared with direct hybridization to membrane filters (for comparison, see *Bornkamm* et al., *Pettersson* et al., this volume; *Brandsma* and *Miller* 1980). Because of the simplicity of sample treatment, i.e., boiling in SDS, the hybridization test can be carried out without the delay caused by immobilization of the sample on a filter. Furthermore, the sample is always treated similarly regardless of whether the nucleic acid to be detected is DNA or RNA. The sensitivity of the test is increased considerably when mRNA produced by the virus can be detected in the test in addition to the viral genome. Finally, sandwich hybridization has turned out to be less sensitive to contaminating materials in the sample than is direct hybridization, in which, e.g., mucus causes background problems.

According to the results presented here and by others in this volume, we think that nucleic acid hybridization will become a useful tool in microbial diagnosis in the future, due to its rapidity and high specificity.

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Insertion and Expression of Viral Information in Tumors

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1 Introduction

It is now well known that the genome of retroviruses contains two groups of genes associated with malignant cell transformation. The first group is constituted by the so-called oncogenes encoding transforming proteins, and the second is a group of regulatory genes which participate in processing and transcription of viral information.

At present more than ten oncogenes have been identified which are either independent genes, that is, containing only unique sequences (*src, ras*) or "fusion" genes containing sequences encoding virion structural proteins (*gag* or *pol*) as well as unique sequences (*myc, myb, erb, rel, abl, ros, mos, sis, fes, fms, bas*) (*Coffin* et al. 1981).

Regardless of its contents, this gene group encodes the so-called oncogene (*onc*) proteins, among which some have a kinase activity that phosphorylates tyrosine in certain cell proteins (*Collett* and *Erikson* 1978).

In animals with tumors induced by sarcoma viruses, antibodies are synthesized which immunoprecipitate *onc* proteins (*Brugge* and *Erikson* 1977) Fusion *onc* proteins contain *gag* as well as unique determinants, thus opening up possibilities for detecting these proteins with antisera to the corresponding virion structural proteins (*Hanafusa* et al. 1980).

2 Role and Demonstration of Oncogene Sequences

Two sets of data obtained in studying oncogenes are of particular significance. First, it was discovered that oncogenes are highly conserved, and *onc* sequences similar to

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those in viral genomes could be detected in the genomes of normal cells at different levels of phylogenetic development (*Bishop* 1981). Second, within the regulatory genes promotor sequences were identified that could "work" not only with viral, but with cellular genes as well (*Yamamoto* et al. 1981). Therefore it might be presumed that in many tumor cells malignant transformation could be initiated and maintained either by exogenous or endogenous sequences.

In principle, there are four possibilities for joint action of promotor and *onc* sequences:

1. Exogenous infection in which both viral genetic elements neighboring each other are integrated into the host cell genome
2. Insertion of exogenous promotor sequences (perhaps of viral origin) in a position near the cell oncogene
3. Rearrangement of internal sequences in host cell DNA as a result of action by different modification agents
4. Specific integration of an exogenous oncogene (of viral origin?) in the neighboring position to the cell promotor sequence

Unfortunately, not all of the possibilities mentioned above could be tested. The main reason for this is that only a few oncogenes have been isolated, and it is not always possible to synthesize the corresponding radioactive probe to detect oncogenes and promotors. However, some modern methods have been developed for the detection of oncogenes and their products. Among these, the most convenient are the genetic engineering methods which have led not only to the production of bacterial clones containing inserts of all the known oncogenes, but also to methods of identifying these genes in cellular DNA and RNA (i.e., the Southern blot hybridization method; *Southern* 1975). Another useful method is that of transfection. Using this technique *Oskarsson* et al. (1980) showed that ligation of promotor sequences to an oncogene greatly increases the transformation potential over that of the oncogene alone, whereas *Shilo* and *Weinberg* (1981) demonstrated the possibility of inducing a transformed phenotype in NIH 3T3 mouse cells after transfection with DNA from human bladder cancer cells.

3 Model Experiments with Rous Sarcoma Virus-Induced Tumors

In model systems (i.e., mammalian cell lines obtained from Rous sarcoma virus [RSV]-induced tumors) the viral genome could be detected by blot hybridization (Fig. 1). The data presented show that three cell lines transformed by RSV have different types of virus-specific *Eco*RI fragments in their genomic DNA. It is important to note that the DNA of all the cell lines yields only one common RSV-specific fragment upon digestion. That this fragment contains *src* gene sequences was confirmed by hybridization with an *src*-containing plasmid as probe (Fig. 2). Thus on the basis of these experiments it is possible to conclude that one functioning oncogene is enough to maintain the transformed phenotype.

Although many facts confirm the proposition that the oncogene is the main transformed phenotype-controlling factor, some other data lead us to suppose that the infecting viral oncogene could perform some other role as well. Murine RVP-3 cells could be taken as an example. This cell line was obtained from murine tumors

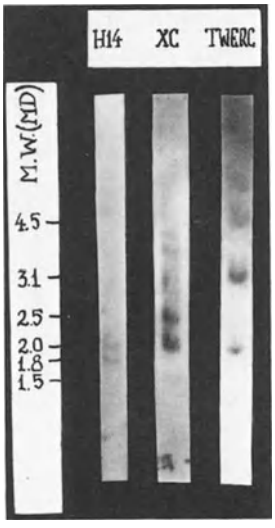

Fig. 1. Distribution of virus-specific sequences in DNA of different RSV-transformed mammalian cell lines of hamster (*H14*) and rat (*XC* and *TWERC*) origin. High molecular weight cellular DNA was digested with *Eco*RI and analyzed by Southern blotting using [^{32}P]cDNA of RSV. *MW*, molecular weight; *MD*, megadaltons

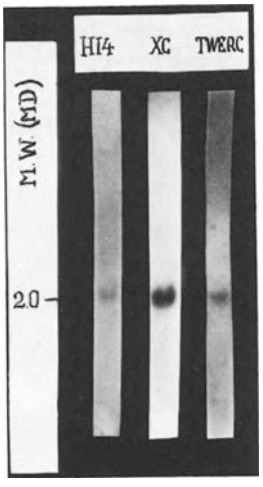

Fig. 2. *src*-specific sequences in DNA of transformed cells (as indicated in Fig. 1). Nick-translated [^{32}P] DNA synthesized on pBR322 containing the *src* insert was used as radioactive probe. *MW*, molecular weight; *MD*, megadaltons

induced by RSV, and during the initial passages in vitro expression of the viral genome was well defined. During passaging of these cells in vitro gradual decrease of RSV expression was observed, and at present no traces of virus expression can be detected (Fig. 3). Using the blot hybridization technique we were also not able to detect any RSV-specific proviral sequences in the cell genome. It should be emphasized that throughout the time of passaging, RVP-3 cells retained the transformed phenotype and induced tumors in vivo (*Tatosyan* et al. 1980). On the basis of these data we supposed that the RSV genome could activate an endogenous oncogene whose activity could be responsible for the transformed phenotype of RVP-3 cells. Expression of the *mos* and *abl* genes was tested for in these cells, but the results were negative for both genes. The possibility of endogenous oncogene activation by the insertion of an exogenous viral promotor was first demonstrated by *Hayward* et al. (1981). Later, these results were confirmed by the work of Payne et al. (1982), but a diffi-

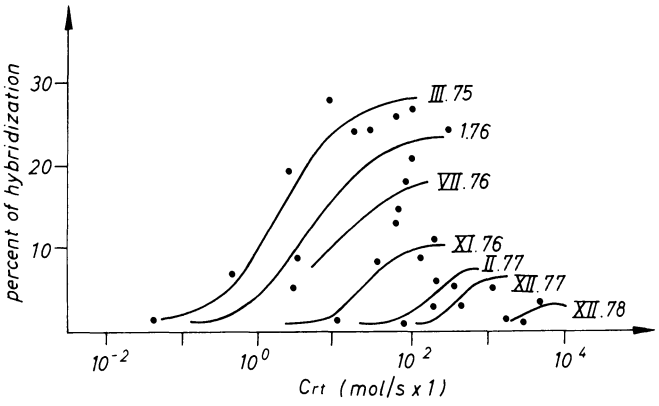

Fig. 3. Gradual decrease of RSV expression in the transformed RVP-3 cell line. Total cellular RNA was hybridized with RSV [^{3}H]cDNA synthesized in the endogenous reverse transciptase reaction

cult-to-explain result was obtained. It appeared that the viral promotor in bursal lymphomas could be joined to the *myc* gene on either side, and it enhanced its activity in both cases (*Payne* et al. 1982).

Summarizing the present data, it can be concluded that in tumor cells, both exogenous and endogenous oncogenes can function. These oncogenes can be activated as a result of interaction with promotor sequences. Therefore, the main questions are how to identify functioning oncogenes in tumor cells, and whether functioning oncogenes are an indication for tumor cells. It is possible that in some systems oncogene activation may take place in differentiating cells, and a correlation between oncogene expression of a given cell type and cell origin is absent (*D. Stehelin*, personal communication). It would seem, therefore, that oncogenes could play an important role not only in oncogenesis, but during differentiation as well.

4 Oncogene Expression in Human Tumor Cells

The data obtained in model systems has made it possible to study oncogene expression in human tumor cells. These experiments were based on the fact that the nucleotide sequences of oncogenes in normal cells are highly conserved. This problem has recently been analyzed in more detail in the work of *Eva* et al. (1982), in which the expression of four oncogenes (*sis, myc, abl,* and *bas*) in lines of human tumor cells was investigated. For the *sis* gene, expression was detected in 8 out of 23 tumors tested; for the *myc, bas* and *abl* genes, expression was detected in all tumor and normal cells. As a rule, the transcripts of these oncogenes have different and specific sizes, and there was no correlation between the expression of the different oncogenes and the type of tumor.

Using the dot hybridization technique we monitored the expression of two genes, *src* and *mos*, in human tumors, either passaged in nude mice or in a few cases, taken directly from patients. The results of these experiments are presented in Table 1. It follows from the data presented that RNA specific for both genes could be synthesiz-

Table 1. Detection of *src*- and *mos*-specific RNA in human tumors by dot hybridization[a]

Type of tumor	Type of [^{32}P]nick-translated probe	
	src (Derived from chicken Rous sarcoma virus)	*mos*[b] (Derived from murine Moloney sarcoma virus)
Human tumors adapted to growth in nude mice		
1. Larynx cancer	±	±
2. Stomach cancer	+	–
3. Large-intestine cancer (strain PTK 1)	+	±
4. Large-intestine cancer (strain PTK 2)	±	–
5. Large-intestine cancer (strain PTK 7)	+	+
6. Ewing sarcoma	+	+
7. Nephroblastoma	±	–
Primary tumors from patients		
1. Osteogenic sarcoma	+	+
2. Soft-tissue sarcoma	+	+
3. Soft-tissue sarcoma	–	–
4. Hemangiopericytoma	–	–
5. Fibrous histiocytoma	+	+
Controls		
TWERC cells	++	±
Mouse liver	+	+

[a]First spot contains 20 μg cellular RNA; [b]pBR322 containing the *mos* insert was kindly supplied by Dr. K. Chumakov, Institute of Molecular Biology; USSR Academy of Medical Sciences. Moscow, USSR

ed in human tumor cells. In some cases both genes are activated, whereas in other cases only one is. In tumors of one type obtained from different patients, activation of the two tested genes can differ. Thus neither the authors cited nor ourselves were able to detect any regular pattern in this respect.

5 Conclusions

In summary, the following conclusions can be made. Oncogenes are a special group of genes present in normal cell genomes and in the genomes of viruses with direct oncogenic potential. Malignant cell transformation is induced either after endogenous oncogene activation or after integration of an exogenous oncogene (of viral origin, as a rule). In some cases the oncogenic potential of the virus may be mediated by the integration of only the promotor sequences, which by themselves can activate the host cell oncogene.

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Volume 101

Tumorviruses, Neoplastic Transformation and Differentiation

Editors: T. Graf, R. Jaenisch
1982. 27 figures. VIII, 198 pages. ISBN 3-540-11665-6

Tumor viruses are widely used as tools to analyze the cellular processes which lead to malignant transformation. One of the most intriguing and important aspects of tumor virus-cell interactions is the relationship between the differentiated state of the host cell and its susceptibility to infecting viruses. This book presents a series of reviews dealing with experimental systems in which only cells at a specific stage of differentiation are permissive for viral transformation and/or replication.

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A. Levine reviews evidence showing that the stage of differentiation during early mouse development influences the ability of Polyoma virus to replicate in embryonic cells.
N. Hynes and B. Groner discuss recent knowledge about the interaction between mammary tumor virus sequences and glucocorticoid hormones and their receptors. Finally
R. Smith reviews current knowledge about avian retroviruses causing osteopetrosis and W. Doerfler discusses the mechanisms of integration of Adenovirus DNA into the host cell's genome.

This collection of reviews is intended for the interested student as well as for scientists to familiarize them with some of the most exciting developments in the study of tumor virus-cell interactions.

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Volume 97

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Volume 98

Retrovirus Genes in Lymphocyte Function and Growth

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Volume 100

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A Workshop at the Basel Institute for Immunology
Organized and edited by H.V. Boehmer, W. Haas, G. Köhler, F. Melchers, J. Zeuthen
With the collaboration of S. Buser-Boyd
1982. 52 figures. XI, 262 pages.
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1983. 40 figures. IV, 152 pages
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Volume 103

Retroviruses 1

Editors: P.K. Vogt, H. Koprowski
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ISBN 3-540-121267-6

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A collection of timely reviews on various aspects of retrovirus research is contained in this volume. Written by leading virologists and molecular biologists from the USA and the United Kingdom, the reviews cover the genome structure of endogenous retroviruses, synthesis, integration and transcription of the provirus and the molecular biology of sarcoma and leukemia viruses.

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