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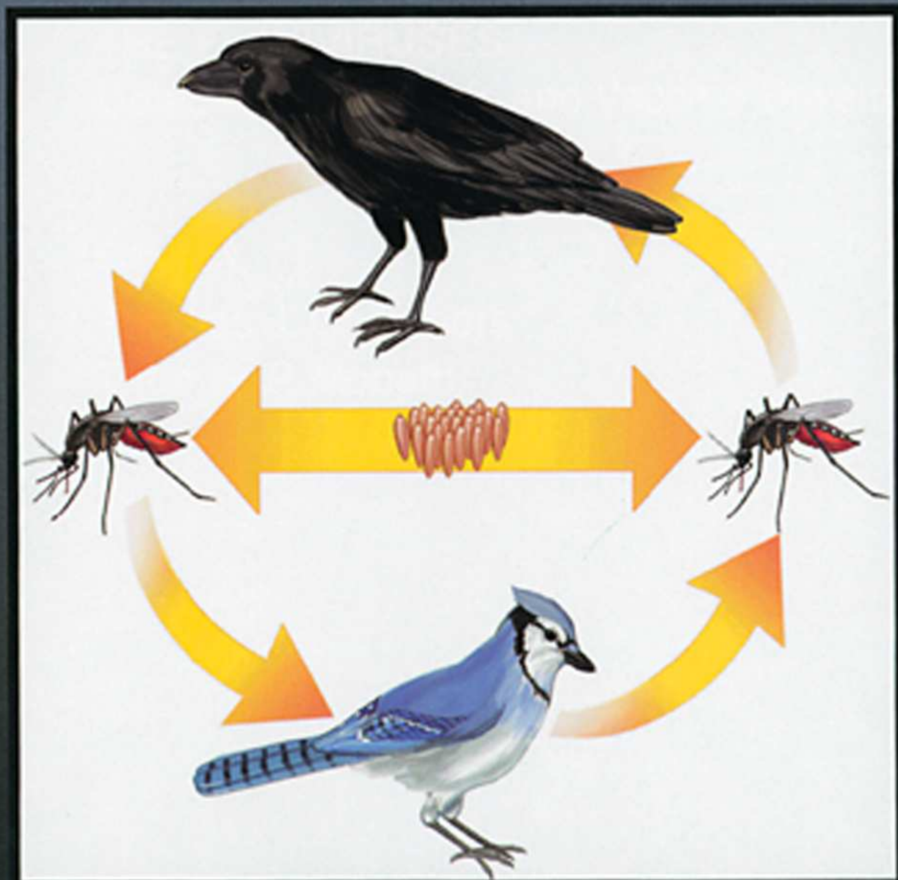
THE FLAVIVIRUSES:

DETECTION, DIAGNOSIS AND VACCINE DEVELOPMENT



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VOLUME 61
The Flaviviruses: Detection, Diagnosis, and Vaccine Development

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PREFACE TO THE FLAVIVIRUSES

The Flavivirus family continues to provide great fascination for virologists, immunologists, entomologists, epidemiologists, and scientists in various other disciplines. Research over the past few decades has yielded considerable progress in many of these areas, but there remain a number of challenges surrounding our understanding of the behavior of flaviviruses in natural conditions and in the laboratory. At a time when continued global emergence of flaviviruses calls for the development and improvement of vaccines and antiviral agents, it is appropriate that a broad compendium of knowledge be made available that presents recent conceptual advances and reviews current information on the many different facets of these viruses. Certainly there have been some noteworthy scientific achievements. For instance, the molecular details of virus structure have been greatly advanced as a result of high-resolution analysis of the envelope protein and its organization at the level of the virion particle, which, together with functional studies, have revealed the uniqueness of this viral protein during replication and pathogenesis. The characterization of an increasing number of flavivirus strains at the sequence level has led to an improved taxonomic classification of the genus, enhanced our understanding of evolution, geographic variation, and epidemiology, and stimulated research on variation in viral virulence. Use of molecular clone technology has advanced from basic studies that have that have identified the functions and properties of viral proteins during RNA replication and virus assembly to the evaluation of candidate virulence determinants, engineering of live attenuated vaccines, and related applications.

Studies on the immunobiology of flaviviruses have led to the realization that these viruses interact with the host immune system in ways that differ from other small RNA viruses. The importance of neutralizing antibody responses for immunity continues to be an area of focus, and the basis for this protection at the epitope-specific level is gradually being dissected. However, there remain enigmatic aspects, such as the wide cross-reactivity elicited by these viruses and the phenomenon of antibody-dependent enhancement, both of which have important implications for pathogenesis and vaccine development, and

require better molecular characterization. It is becoming clear that T-cell responses to flavivirus infections also have unusual properties that may contribute to pathogenesis through immunopathologic and/or immune-subverting events. Further characterization of these responses and their relationship to immune protection are avenues of research needed to optimize the use of the increasing range of vaccine modalities that are being pursued.

In conjunction with advances in flavivirus molecular virology and immunology, more and more attention is being directed to investigation of the pathogenesis of flavivirus diseases. Progress in this area has been heralded by the long-awaited identification of the molecular basis for genetic susceptibility of mice to flaviviruses. This will undoubtedly increase interest in the role of innate defenses in these infections and promote research into the genetic basis of flavivirus susceptibility in humans. Together with the use of modern techniques to identify critical target cells of infection, research in this area will expand our understanding of the cellular and molecular basis for flavivirus tropism. In this regard, the cell-surface molecules that interact with these viruses during entry have yet to be fully characterized, but progress continues to be made on this front. It remains somewhat frustrating that suitable animal models for some flavivirus diseases, particularly dengue hemorrhagic fever, are not available. However, data accumulated from human clinical studies are yielding insight into the pathogenesis of this disease, and similar studies with other pathogenic flaviviruses are anticipated in the future.

The interactions between flaviviruses and their arthropod hosts have been the subject of many classical studies that have now progressed to the molecular level as well. There are many secrets to these interactions that must be discovered to understand the process of virus persistence in molecular terms. These will be forthcoming with the use of modern technologies by creative investigators interested in vector biology. The improvement in molecular technologies has had concomitant impact on the ability to conduct molecular epidemiology at the “macro” and “micro” levels. In response to progressive emergence in recent years of dengue, Japanese encephalitis, West Nile, and tick-borne viruses, the application of such technologies for detection and surveillance in arthropod and vertebrate reservoirs has provided insight into the factors that support the global movements of flaviviruses. Yet, there is a tremendous amount of such data concerning virus evolution in the natural environment that is still needed to understand this process and possibly predict future

trends. Additional molecular studies of these viruses as they are transmitted among vectors, reservoirs, and humans are needed to further our conceptual understanding of virus emergence.

The development of vaccines for flaviviruses has also benefited greatly from the availability of modern technologies, and new as well as next-generation vaccines for some viruses are on the horizon. As better understanding of the immune responses to these viruses in the context of disease as well as vaccine-induced protection becomes available, the ability to control the growing worldwide burden of disease from these agents will likely be improved.

Clearly a comprehensive research approach in many scientific disciplines is needed to unravel the complexities of the virus-host interactions that these viruses have had the benefit of manipulating for centuries. In this three-volume edition on the flaviviruses, our goal has been to assemble a base of knowledge that encompasses these complexities, describes technologies that have contributed to this knowledge, and identifies the major problems faced in attempting to further understand the virus-host interactions that result in disease, and in using vaccine strategies for preventing them. We are grateful to the many contributors who have generously assisted in the preparation of this book series. We must also acknowledge that there are many other colleagues who are active in the field whose expertise has not been represented here.

Thomas J. Chambers,
St. Louis, Missouri, 2003

Thomas P. Monath
Cambridge, Massachusetts, 2003

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PREFACE TO VOLUME 61
THE FLAVIVIRUSES: DETECTION, DIAGNOSIS, AND VACCINE DEVELOPMENT

Emergence of flaviviruses is a continuing reality and a threat to public health on a worldwide scale. Movement of many of these viruses both regionally and globally has been occurring progressively for the last half-century, resulting in new distributions of viruses, new vector associations, and new human disease syndromes. Japanese encephalitis virus has penetrated many areas in Asia, including the Australasian region to the east and parts of India and Pakistan to the west. Dengue has established itself as a pandemic virus. West Nile virus has both evolved and emerged as the most important cause of arbovirus encephalitis in the Western hemisphere. Growing burdens of tick-borne flavivirus diseases in their endemic zones also pose concerns for regional public health as well as intercontinental spread. Proliferation and dissemination of arthropod vectors, long-range human travel and bird migration, human incursions into vector and reservoir habitats, and environmental disturbances are major factors in the ongoing emergence of these viruses that deserve consideration for control efforts. However, better knowledge of actual vector-host cycles and how they evolve into epidemic outbreaks in conjunction with sensitive molecular and serological assays for detection and diagnosis of these viruses will also contribute to the ability to assess their potential for future transmission.

Despite many possible avenues toward prevention and control of flavivirus diseases, vaccine development still offers the most promising approach. Although yellow fever 17D serves as a paragon for live-attenuated viral vaccines, adopting this modality for other serious flavivirus pathogens has not been very straightforward, with the multiple serotypes of dengue virus being the most flagrant example. Achieving a suitable balance of attenuation and immunogenicity remains a difficult proposition with live viral vaccines, and safety issues continue to be prominent, even for YF 17D. Inactivated vaccines have traditionally also been effective against some flaviviruses, but seem destined for replacement by subunit vaccines designed to have better immunogenicity and fewer side effects. The use of alternative

technologies, including DNA vaccines for flavivirus, is under active investigation, with an as yet undefined role in the next generation products. Demand for flavivirus vaccines encompasses military, civilian, and veterinary realms, and has increased because of the threat of bioterrorism. Development of new vaccines will exploit innovations that arise from continued basic research on flavivirus evolution, biology, and pathogenesis. The challenge of keeping pace with these viruses in the future will certainly remain, despite advances in these many different areas.

Thomas J. Chambers
Thomas P. Monath

DIAGNOSIS AND SURVEILLANCE

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ADVANCES IN VIRUS RESEARCH, VOL 61

SERODIAGNOSIS OF FLAVIVIRAL INFECTIONS AND VACCINATIONS IN HUMANS

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I. Introduction

In the past three decades, we have witnessed a considerable technological shift and a dramatic proliferation of commercially available reagents, diagnostic kits, and testing services for serodiagnosis of flaviviral infections or vaccinations. As more diagnostic data were

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produced, ironically the inadequate levels of our understanding of the variation in antibody response, cross reactivity of antibody, and of other associated complications involved in diagnostic practice became more evident. In addition, new molecular and enzyme-linked immunosorbent assay (ELISA) techniques revealed that some of the cases previously classified as negative or not current infections based on the conventional serologic criteria were found to be actually current infections. In this chapter, the principles and applications of both traditional and new techniques are first critically re-evaluated, followed by an examination of the qualities of specimens. Variations of human antibody response kinetics are briefly summarized, because a clear understanding of the subject is indispensable for a better serodiagnosis. Then, miscellaneous diagnostic complications and the qualities of the criteria used in common serologic techniques are examined. Finally, a list of commercial reagents, diagnostic kits, and services is presented.

Basically, serodiagnosis is performed for one of the three major objectives: laboratory diagnosis of an ill patient, a seroprevalence study in an epidemiologic investigation, and to evaluate an immune response in humans and animals, including vaccine efficacy trials and animal experiments. As more vaccines are introduced, for those in public health, epidemiology, veterinary medicine, and agriculture, it has become critical to be able to differentiate the antibodies induced upon vaccination from those acquired in response to natural infection and homologous *in vivo* protective antibodies from heterologous, nonprotective *in vitro* neutralizing antibodies, for designing an efficient immunization strategy for a particular human (or animal) population and for determining the safety of importing or exporting animals.

II. Serodiagnostic Tests

The techniques based on specific agglutination of blood cells, bacteria, or particles had been developed early in the history of flaviviral serodiagnosis. With the advent of ELISA, some of the traditional techniques, in particular, the complement fixation test (CF), lost popularity, and radioimmunoassay is no longer practiced in arbovirology. Nevertheless, in terms of the overall advantages and other benefits, the hemagglutination-inhibition (HI) test still remains invaluable and the neutralization test (NT) most specific for primary infections.

A. Standardization and Multicenter Evaluation of Serologic Tests

Unquestionably, standardization of key serologic tests at global or at least at the regional level for those viral diseases affecting many countries (such as DEN, JE, TBE, WNF, and YF) is highly desirable, although past such attempts have met a variety of difficulties. With the increasing trends toward the use of commercial reagents, kits, and services (Table I), occasional multicenter evaluation of selected tests is another approach for reducing variation in diagnostic quality among institutions. In all these activities, participation of governmental laboratories, research institutions, and industry is of critical importance.

B. Validation and Quality Assurance

For each serologic test, at least a pair of negative and positive control human serum specimens must be obtained from a reliable reference laboratory or other sources, if not readily available. Preferably, they are pooled, laboratory-confirmed specimens when many tests are planned or expected. When no source of such control specimens exists or it is difficult to obtain, at least internal controls must be prepared in each laboratory from the specimens of laboratory-confirmed cases. In the highly *Flavivirus*-endemic locations where negative control specimens are not easily available, acquisition of serum specimens from the residents in the non-endemic areas should be arranged. These control specimens must be tested independently in each laboratory at first using the same technique used in the source laboratory, and necessary adjustments are made until the results in the two institutions are comparable. If a different technique is used, a comparative test between the two methods must be performed to evaluate the qualities of the results obtained with the different techniques. In each test, routinely this pair of control specimens is included and their titers monitored within a test or between tests for quality control. If available, additional internal control specimens may be included. For interpretation of results, first, a set of diagnostic criteria are established, including the acceptable range of variation from the optimal results with negative or positive control specimen. For any specimens of special importance, it is ideal to perform more than one diagnostic test, even including non-serologic tests, for improved reliability of the diagnostics. In addition, it is strongly recommended that all diagnostic laboratories or institutions, regardless of the depth of experience, arrange a periodic proficiency test program in collaboration with an unaffiliated,

TABLE I
LIST OF COMMERCIALLY AVAILABLE REAGENTS, DIAGNOSTIC KITS, AND TESTING SERVICES FOR FLAVIVIRAL INFECTIONS[a]

Virus	Product[b]	Source[c]
DEN	Ag DEN 1-4	ANS/FII/MIB/USB
	DEN-1	ANS/BID/BGN/IMC/VNT
	DEN-1 fENV	VNT
	DEN-1 rENV	HWB
	DEN-2	ANS/BGN/BID/FII/IMC/MIB/VNT
	DEN-2 (16681)	BID/MIB
	DEN-2 fENV	VNT
	DEN-2 rENV	HWB
	DEN-3	ANS/BID/BGN/IMC/VNT
	DEN-3 fENV	VNT
	DEN-3 rENV	HWB
	DEN-4	ANS/BID/BGN/IMC/VNT
	DEN-4 fENV	VNT
	DEN-4 rENV	HWB
	DPS AgC	GLB/MAS/PNB
	ELISA IgMC	AMQ/CBT/CHM/FCT/GLY/GVT/IAC/OMD/PNB
	IgGC	AMQ/CBT/CHM/FCT/GLY/GVT/IAC/OMD/PNB
	IgGI	PNB
	IgGT	PNB
	HYB DEN complex-specific (2H2-9-21)	ATC
	DEN-1 (15F3-1)	ATC
	DEN-2 (3H5-1)	ATC
	DEN-3 (5D4-11)	ATC
	DEN-4 (1H10-6)	ATC
	IFA IgG	AMR/PGB
	IgM	AMR/PGB
	IMB AgC	GLB
	IgG	GLD/VNT
	IgG/IgM	PNB
	IgM	GLD/VNT
	IMC Strip IgG	AMT/GVT/RBP
	IgM	AMT/GVT/RBP
	Cassette IgG/IgM	PNB
	IgG	AMT/CDI/GLY
	IgM	AMT/CDI/GLY

(*continues*)

TABLE I (*continued*)

Virus	Product[b]	Source[c]
	MAb DEN complex reactive	BGN/BID/CHM/USB/VNT/VRS
	DEN 1-4 (IgG & IgM)	IMC
	DEN 1-4 (BD1419)	ACS
	DEN 1-4 (9F14)	BGN
	DEN 1-4 (M125)	FII
	DEN-1	ANS/CGM/ECB/IMC/USB
	DEN-1 (9F10)	BGN
	DEN-1 (M121)	FII/USB
	DEN-1 (15F3)	CHM/MIB
	DEN 1+2 (biotin-labeled)	CMI
	DEN-2	ANS/BID/CHM/ECB/IMC/USB/VNT
	DEN-2 (9F11)	BGN
	DEN-2 (M122)	FII
	DEN-2 (3H5)	CHM/MIB
	DEN-2 (Env-specific)	VNT
	DEN-2 (NS1-specific)	VNT
	DEN-3	ANS/CHM/ECB/IMC/USB
	DEN-3 (9F12)	BGN
	DEN-3 (M123)	FII
	DEN-3 (5D4)	BID/CHM/MIB
	DEN-4	ANS/BGN/CHM/ECB/IMC/USB
	DEN-4 (9F13)	BGN
	DEN-4 (M124)	FII
	DEN-4 (1H10)	BID/CHM/MIB
	PAb DEN-complex reactive	
	(positive control)	PNB
	(mouse)	ATC
	(rabbit)	BGN/BID
	DEN-complex reactive	BID
	IgG (human)	BID/IMC
	IgG (rabbit)	IMC
	IgM (human)	IMC
	anti-DEN-1 (human)	ANS
	anti-DEN-1 (mouse)	ANS/ATC
	anti-DEN-1 (rat)	ANS
	anti-DEN-2 (human)	ANS
	anti-DEN-2 (mouse)	ANS/ATC
	anti-DEN-3 (human)	ANS
	anti-DEN-3 (mouse)	ANS/ATC

(*continues*)

TABLE I (*continued*)

Virus	Product[b]	Source[c]
	anti-DEN-3 (rat)	ANS
	anti-DEN-4 (human)	ANS
	anti-DEN-4 (mouse)	ANS/ATC
	anti-DEN-4 (rat)	ANS
	Service DEN IgG ELISA	ARUP/FCT
	DEN IgM ELISA	ARUP/FCT/SPL
	DEN total Ab (CSF)	SPL
	Virus[d] (live & inactivated)	
	DEN-1,2,3,4	ANS/ATC
	DEN-2	BID/USB
Flavi-viruses	HYB Group reactive (4G2)	ATC
	IFA Arbovirus screening Including Flaviviruses	PNB
	MAb Group reactive (4G2/6B6C-1)	CHM/HRA/MIB
	Service Diagnostic–unspecified	QST
	Custom MAb production	CMI
JE	Ag rEnv	HWB
	rPrME	MIB
	CF	ACS/DSC
	HI	ACS/DSC/KYB
	ELISA IgM	VNT
	IMB IgM	VNT
	MAb JE-specific (995)	MIB
	6B4A-10 (also SLE, MVE, WN-reactive)	CHM
	Group-reactive	VNT
	Envelope-specific	VNT
	NS1-specific	VNT
	PAb (mouse)	ATC
	CF (mouse)	ACS/DSC
	HI (mouse)	ACS/DSC
	Service IgM (serum)	SPL
	IgM (CSF)	SPL
	Virus[d]	ATC
KUN	PAb	ATC
(also see WN)	MAb (10A1)	CHM
MVE	MAb (4B6C-2)	CHM

(*continues*)

TABLE I (*continued*)

Virus	Product[b]	Source[c]
RSSE	PAb (mouse)	ATC
	(sheep)	NIBSC
SLE	DPS AgC	MAS
	MAb (1B5D-1/6B5A-2)	CHM
	PAb (mouse)	ATC
	Service IgG/IgM IFA	ARUP/CNI/FCT/QST/SPL/VRM
	Virus[d]	ATC
TBE	Ag (inactivated)	ANS (permit required)/SID
	ELISA IgG (human)	DBM/EUI/GLY/GVT/PGB/SID
	IgM (human)	DBM/EUI/GLY/GVT/PGB/SID
	(for animals)	PGB
	PAb (human)	ANS
	PAb	
	Int. Ref. Reagents for TBE against louping ill strain	NIBSC
	against Sofjin and Absettarov strains (sheep)	NIBSC
WN	Ag (gamma irradiated)	
	(for avian serology only)	BRC
	(for equine serology only)	BRC
	rPrME	FCT/HRA
	DPS AgC	MAS/PNB
	ELISA IgG and IgM	IBS/PNB
	IFA IgG (slide)	PNB
	MAb (WN-A or H546)	BID/MIB
	Env	CHM
	Nt Ab to Env	BRC
	Non Nt to Env.	BRC
	(for IFA and IHC)	BRC
	PAb (mouse)	ATC/BRC
	(positive control)	PNB
	(avian spp. IgG)	BRC
	(equine IgG+IgM)	BRC
	(rabbit IgG for IFA & IHC)	BRC
	Service IgG/IgM ELISA or IFA	ARUP/FCT/QST/SPL/VRM
	Virus[d]	ATC
YF	HYB (2D12)	ATC/CHM/EUR
	MAb (2D12A)	CHM
	(OG5)	BGN
	(2031–13)	BGN

(*continues*)

TABLE I (*continued*)

Virus	Product[b]	Source[c]
	17D-specific (864)	MIB
	Wild-strain specific (117)	MIB
	PAb (mouse)	ATC
	(monkey) [WHO International Reference]	NIBSC
	Virus[d]	ATC

Other viruses (and their corresponding mouse ascitic fluids-AF) at ATC: Banzi; Bukalasa Bat; Bussuquara (AF); Cowbone Ridge (AF); Dakar bat; Edge Hill; Entebbe bat; Ilhéus (AF); Kokobera; Modoc (AF); Montana myotis meningoencephalitis (AF); Murray Valley encephalitis; Ntaya; Powassan (AF); Rio Bravo (AF); Sepik; Stratford; Tembusu (AF); Uganda S; Zika.

[a] Mention of trade names and sources is for identification only and does not imply the endorsement by the Centers for Disease Control and Prevention or U.S. Dept. Health and Human Services. The list includes, in addition to the original manufacturers, the corporations that only market the products made by the others. Product availability is valid as of April 2003 but subject to rapid change depending on market conditions.

[b] Product abbreviations. Ag: antigen; AgC: antigen capture; Cassette: horizontal flow card test; CF: complement fixation; DPS: dipstick; EIA: enzyme immunoassay (unspecified); ELISA: enzyme-linked immunosorbent assay; ENV: envelope protein; fEnv: envelope protein as fusion protein; HI: hemagglutination inhibition; HYB: hybridoma cell; IFA: immunofluorescence; IgGC: IgG capture; IgGI: IgG indirect; IgGT: IgG total; IgMC: IgM capture; IHC: immunohistochemistry; IMB: immunoblot/immunodot; IMC: immunochromatographic test; MAb: monoclonal antibody; Nt: neutralizing; PAb: polyclonal antibody; rEnv: recombinant envelope protein; rNS1: recombinant NS1 protein; rPrME: recombinant PrM-E protein; Service: testing or custom service.

[c] Product sources. (E: e-mail address; F: fax number; W: website [after http://www.])

ACS: Accurate Chemical & Scientific (Westbury, NY, USA)
E: info@accuratechemical.com F: +1 516-997-4948
W: accuratechemical.com

AMQ: American Qualex Antibodies, Inc. (San Clemente, CA, USA)
E: info@americanqualex.com F: +1 949-492-6790

AMR: American Research Products (Belmon, MA, USA)
E: staff@arp1.com F:+1 617-489-5120 W: arp1.com

AMT: AmeriTek, Inc. (Seattle, WA, USA)
E: info@ameritek.org F:+1 206 528-8107
W: ameritek.org

ANS: Antibody Systems, Inc. (Bedford, TX, USA)
E: asitmf@airmail.net F:+1 817-498-8277 W: antibodysystems.com

ARUP: ARUP Laboratories (Salt Lake City, UT, USA)
F: +1 801-583-2712 W: aruplab.com

ATC: American Type Culture Collection (Manassas, VA, USA)
E: sales@atcc.org F: +1 703-365-2750 W: atcc.org

BGN: Biogenesis Ltd. (Poole, UK)
E: biogenesis@sprintmail.com F: +44 1202660020
W: biogenesis.co.uk

(*continues*)

TABLE I (*continued*)

[In USA, Biogenesis, Inc. (Brentwood, NH, USA)]
BID: Biodesign International (Saco, ME, USA)
E: info@biodesign.com F: +1 207-283-4800 W: biodesign.com
BRC: BioReliance Corp. (Rockville, MD, USA)
E: bpeters@bioreliance.com F: +1 301-838-0371 W: bioreliance.com
CBT: Calbiotech, Inc. (Spring Valley, CA, USA)
F: +1 619-660-6970 W: calbiotech.com
CDI: Cortez Diagnostics, Inc. (Calabasas, CA, USA)
E: onestep@rapidtest.com F: +1 818 591-8383
W: rapidtest.com
CHM: Chemicon International, Inc. (Temecula, CA, USA)
E: custserv@chemicon.com F: +1 809-437-7502; W: chemicon.com
CMI: Custom Monoclonals International (West Sacramento, CA, USA)
E: ckgrantcmi@rcip.com F: +1 916-372-3329 W: cmi.rcip.com
DBM: Dade Behring Marburg (Marburg, Germany)
W: dadebehring.com
DSC: Denka Seiken Co., Ltd. (Tokyo, Japan)
E: seikei2@denka-seiken.co.jp F: +81 3-669-9390
ECB: East Coast Biologicals, Inc. (Berwick, ME, USA)
E: info@eastcoastbio.com F: +1 207-676-7658 W: eastcoastbio.com
EUI: Euroimmun (Lübeck, Germany)
E: info@euroimmun.de F: +49 4509 874334 W: euroimmun.de
EUR: European Collection of Cell Culture (Salisbury, UK)
F: +44 1980612511 W: camr.org.uk/ecacc.htm
FCT: Focus Technologies (Cypress, CA, USA) [formerly Microbiology Reference Lab]
F: +1 714-220-9213 (Test Service)// F: +1 714-220-1820 (Products)
W: focusanswers.com/
FII: Fitzgerald Industries International (Concord, MA, USA)
E: antibodies@fitzgerald-fii.com F: +1 978-371-2266
W: Fitzgerald-fii.com
GLB: Globio Corp (Beverly, MA, USA)
E: info@globio.com W: globio.com
GLD: Genelabs Diagnostics (Redwood City, CA, USA)
E: Jolene@genelabs.com F: +1 650-369-6154 W: genelabs.com
GLY: Glysby, Snc. (Arcore, Italy)
E: glysby@tin.it F: +39 2-688-2269
W: glysby.com (or W: diagnosticworld.com)
GVT: Genzyme Virotech GmbH (Russelsheim, Germany)
E: info@virotech.de F: +49 (0) 61428262-1 W: virotech.de
HRA: Hennessy Research Associates, LLC (Shawnee, KS, USA)
E: khennessy@hennessyresearch.com F: +1 913-268-6195
W: hennessyresearch.com
HWB: Hawaii Biotechnology Group, Inc. (Aiea, HI, USA)
E: info@hibiotech.com F: +1 808-487-7341 W: hibiotech.com
IAC: Immunoassay Center (Havana, Cuba)
E: drdirector@cie.sld.cu F: +53 7-286514
IBS: InBios International, Inc. (Seattle, WA, USA)
E: info@inbios.com F: +1 (206) 344-5823 W: inbios.com

(*continues*)

TABLE I (*continued*)

IMC: Immunology Consultants Lab., Inc. (Sherwood, OR, USA)
E: iclgleslie@aol.com F: +1 503-625-1660 W: icllab.com
KYB: Kyoto Biken (Uji, Japan)
E: fvgk8253@mb.infoweb.ne.jp F: +81 774-24-1407
Lab Corp. (See **QST or VRM)**
W: labcorp.com
MAS: Medical Analysis Systems, Inc. (Camarillo, CA, USA)
E: kdave@mas-inc.com F: +1 805-383-8260 W: vectest.com
MIB: Microbix Biosystems (Toronto, Ontario, Canada)
E: customer.service@microbix.com F: +1 416-234-1626
W: microbix.com
NIBSC: National Institute for Biological Standards and Control (South Mimms, Herts, UK)
E: Standards@nibsc.ac.uk F: +44 1707654753 W: nibsc.ac.uk
OMD: Omega Diagnostics (Alloa, Scotland, UK)
E: odl@omegadiagnostics.co.uk F: +44 0-1259-723251
W: omegadiagnostics.co.uk
PNB: PanBio Pty, Ltd. (Windsor, Queensland, Australia)
F: +61 7-335-71222 W: panbio.com.au
(In the US, Columbia, MD. F: +1 410-381-8984)
PGB: Progen Biotek GmBH (Heidelberg, Germany)
F: +49 6221-403535 W: progen.de
QST: Quest Diagnostics, Inc. (29 locations in the USA)
W: questdiagnostics.com
RBP: R-Biopharm, Inc. (Darmstadt, Germany)
E (for info): webmaster@r-biopharm.com
E (for sales): sales@r-biopharm.com F: +49 (616) 789-3070
W: r-biopharm.com
SID: Serion Immunodiagnostica GmbH (Würzburg, Germany)
E: dialog@virion-Serion.de F: +49 931 52650 W: virion-serion.de
SPL: Specialty Laboratories (Santa Monica, CA, USA)
E: specialty@specialtylabs.com F: +1 310-828-6634
W: specialtylabs.com
USB: United Biological (Swampscott, MA, USA)
E: chemicals@usbio.net F: +1 781-599-9383 W: usbio.net
VNT: Venture Technologies SDN BHD (Sarawak, Malaysia)
E: phtio@mailhost.unimas.my
VRM: Viromed Laboratories (Minneapolis, MN, USA)
E: Clientserv@viromed.com F: +1 952-939-4012 W: viromed.com/
VRS: Virostat (Portland, ME, USA)
F: +1 207-856-6864

[d] Restricted to the qualified institutions in the United States only. Also, in the United States, domestic or international shipment of infectious agents is subject to the latest regulations of the Department of Commerce.

qualified institution or organization. By such an arrangement, a set of coded serum specimens representing negative, low, intermediate, and high titers of antibody received from a collaborating laboratory are tested and the results returned to the sending laboratory for performance evaluation. It is important to include a disproportionately larger number of confirmed positive specimens with low antibody titers in this set of coded specimens, because the quality of diagnostic performance is more accurately judged on those specimens than on the specimens with high titers. This is based on the frequent observations that, using a set of specimens with predominantly only two contrasting titers (negative specimens and positive specimens with high antibody concentrations), the difference in the quality of diagnostic test among laboratories becomes much less evident even if a considerable difference exists (Kuno *et al.*, 1998).

1. Tests Based on Agglutination of Blood Cells or Particles

a. Hemagglutination-Inhibition Test The procedure adopted for microtitration (Sever, 1962) of the original protocol (Clarke and Casals, 1958) has been widely used for a variety of objectives, ranging from case diagnosis to serosurvey.

The principle of this test is based on the propensity of most arboviruses to aggregate erythrocytes of certain animals. If, however, virus is mixed with a serum specimen containing an antibody against the virus, hemagglutination is abrogated, the highest serum dilution causing the inhibition corresponding to the antibody concentration in the specimen. Because hemagglutination is pH dependent, selection of an optimal pH is critical.

The major advantages of the this test are (i) it does not require expensive equipment or instruments and (ii) it is highly useful to initially screen etiologic agents at the major group level because of its extensive and exclusive cross-reaction to all members of one virus group (antigenic complex, genus, or family) and excellent ability to segregate that group from others.

Although it has been sometimes erroneously believed to be an IgG assay, actually it measures other immunoglobulins, such as IgM and IgA, as well. Kaolin treatment of serum specimens for removal of nonspecific inhibitors still leaves a considerable amount of HI-reactive IgM (Granström *et al.*, 1978; Wiemers and Stallman, 1975), although it was once thought to remove it (Mann *et al.*, 1967).

For the visualization of agglutination, goose erythrocytes have been used in most laboratories. Other investigators have found trypsinized human type "O" blood cells or goose cells preserved with formalin

treatment useful in laboratories where fresh goose blood cells are difficult to procure (Ahandrik *et al.*, 1986). As for antigen, sucrose-acetone extracts of infected suckling mouse brains were popularly used in the past, but some of them have been replaced with antigens prepared from infected cell cultures. More recently, recombinant antigens, such as Japanese encephalitis (JE) viral antigen expressed as extracellular subviral particles, became available. However, although some recombinants had a hemagglutination activity (Heinz *et al.*, 1995; Hunt *et al.*, 2001; Konishi *et al.*, 1996), other recombinant antigens either have not been evaluated for utility in the HI test or were found to be nonreactive (Davis *et al.*, 2001; Konishi *et al.*, 2001). Availability of a good HI-reactive recombinant antigen for the diagnoses of West Nile fever (WNF) and other viral infections in wildlife is important, because an HI test with such a safe antigen obviates development of antispecies antibodies necessary in the popular ELISA but currently unavailable commercially.

Although HI antibodies in neurotropic flaviviral infections, compared with those of non-neurotropic infections, are sometimes detectable within 3 to 5 days after the onset of illness because of longer intervals between infection and development of symptoms; generally, a disadvantage of this test is that for case diagnosis it is essentially a retrospective diagnostic test because both acute phase and convalescent phase specimens must be obtained to determine a significant change in antibody titer. Many recovered former patients do not feel a strong need to return to clinics for second blood samples; thus, unless convalescent phase specimens are actively sought by physicians or diagnostic laboratories, many cases with only acute phase specimens would remain inconclusive. Furthermore, it is one of the most cross-reactive tests to flavivirus. It should also be remembered that in the microHI test, which is the standard today, titers obtained are often lower compared with those by the macroHI test (Akov, 1976).

b. Hemadsorption Immunosorbent Test In this test, first, a solid phase (multi-well plate) is sensitized with a capture antibody (such as anti-human IgM antibody). Serum specimen and antigen are added in that order, with washing between steps. When goose erythrocytes are added, hemagglutination develops only in the wells with bound antigen. The hemadsorption immunosorbent test (HIT) has been used for IgM assay for dengue (DEN) and Wesselsbron viral infections (Baba *et al.*, 1999; Gunasegaran *et al.*, 1986). Although, like the HI test, no expensive equipment is necessary for the test, it is not as

sensitive as IgM capture ELISA. Furthermore, a prozone tends to develop in antibody- or antigen-excess regions.

c. Complement Fixation Test The original protocol developed for the serologic study of yellow fever (YF) virus infections in the 1920s was further improved in the early 1930s, laying the foundation for this classic technique (Davis, 1931; Frobisher, 1931). The protocol for microtitration (Casey, 1965) has been most commonly used. Like the HI test, it is not useful as a rapid test during the acute phase of illness due to the requirement of convalescent phase specimens.

The Complement Fixation (CF) test exploits the unique affinity of complement for antigen-antibody complexes. In this test, cellular antigens on the membrane of erythrocytes are complexed with an antibody prepared against the blood cells, and those sensitized cells serve as indicator. In practice, two sets of reagent mixes are prepared. In one set, virus and serum specimen are mixed, to which complement is added later. If the serum had antibody to the virus, complement is fixed to the virus-antibody complex, and little complement remains unbound. In the second set, erythrocytes bearing complement receptors are coated with an anti-erythrocyte antibody (hemolysin). When the two sets of reagents thus prepared are mixed, lysis of erythrocytes does not occur with a positive serum specimen because of little unbound complement. On the other hand, the reaction with negative specimen will result in hemolysis because of a large amount of unbound complement. The relatively short half-lives of CF antibodies are useful markers of recent infection. However, the many disadvantages of CF outnumber the advantages. In addition to the slow rise in titer after infection, CF antibody is not induced in some individuals in any sizable population (Buescher *et al.*, 1959; Doherty *et al.*, 1976). In an SLE outbreak, between 20% and 22% of the patients with confirmed cases did not demonstrate CF antibody 3–8 weeks after onset (Calisher and Poland, 1980). Similarly, the lack of CF antibody response among YF (17D) vaccinees has been well recognized (Monath *et al.*, 1980). Furthermore, contrary to the general belief, reports of persistence of CF antibody for longer than 5 years have not been rare for some flaviviral infections (Buescher *et al.*, 1959; Fujita *et al.*, 1979; Halstead, 1974). Also, some serum specimens are anticomplementary, and hemolyzed blood specimens cannot be used. Most importantly, the complexity of the procedure, which requires titrations of at least three reagents (antigen, complement, and hemolysin) for optimization, is technically demanding and requires time-consuming training of diagnosticians.

d. Immune Adhesion Hemagglutination Test Immune adhesion is an adherence of erythrocytes to tripartite immune complexes of virus antigen-antivirus antibody-complement (C1 or $C3_b$) via C3b receptors on erythrocytes. Addition of complement ($C1_{qrs}$) into antigen-antibody immune complex (IC) initiates transformation of C1 to $C3_b$. The conversion of $C3_b$ in the complement pathway is interrupted by the addition of dithiothreitol. Introduction of type "O" erythrocytes bearing $C3_b$ receptors completes the agglutination of tripartites.

The advantages of IAHA over the CF test are that it is more sensitive and consumes less complement. However, limiting the source to type "O" blood cells poses a supply problem, depending on location. Furthermore, serum specimens taken early (<2 weeks after onset of illness) may not be sufficiently reactive because of their lower sensitivity (Inouye *et al.*, 1980).

e. Reverse Passive Hemagglutination Test When the reverse passive hemagglutination (RPH) test is used for detecting antibody, antiviral antibody is chemically bound to erythrocytes. Separately, the serum specimen and virus antigen are mixed, to which is added the antibody-sensitized erythrocyte suspension. The specimen that does not generate hemagglutination is interpreted as positive. The test has been used for the diagnosis of WNV fever (Estival *et al.*, 2001).

f. Single Radial Hemolysis Test In the single radial hemolysis (SRH) test, virus antigen is bound to erythrocytes. The virus-coated erythrocytes and complement are mixed in melted agar and mixed agar solidified in a mold that produces wells in agar. When a serum specimen containing antiviral antibody is introduced to a well in the gel, the antibody radially diffuses into gel. As antibodies diffuse, they meet and form immune complexes with the antigen bound on erythrocytes. Complement immediately adjacent to the complex interacts with the complexes, lysing the cell membrane, which produces a zone of hemolysis. The test has been used for the diagnoses of DEN, JE, and WNF (Chan, 1985; Duca *et al.*, 1979; George and Pavri, 1986; Guzmán *et al.*, 1985).

g. Indirect Hemagglutination Test This test is a simple modification of hemagglutination test. Antigen-sensitized sheep blood cells are reacted with a serum specimen. After proper mixing and incubation, if hemagglutination is observed, the specimen is scored positive. Although the test is very simple, it suffers from variation in the quality of the sheep erythrocytes used (Gupta *et al.*, 1990).

h. Other Tests Using Synthetic, Natural, or Bacterial Particles Many other techniques based on agglutination use either synthetic or natural particles (Latex, silica, gelatin). The principles of those techniques are the modifications of the aforementioned blood cell agglutination tests and are designed either as direct or indirect (passive) tests, depending on the kinds (antigen, antibody) of ligands bound to particles. The key to successful application lies in optimal preparation of sensitized particles with minimum distortion of ligands while maintaining good reactivity. Particle agglutination tests have been developed for the diagnoses of many human viral diseases but have not been popular for experimental studies of arboviral diseases, except for a small number of studies (Jia *et al.*, 2002; Likar *et al.*, 1971; Yamamoto *et al.*, 2000, 2002).

Bacterial agglutination test takes an advantage of certain strains of bacterial cells bearing immunoglobulin (i.e., IgG) receptors. Because not all antibodies captured are virus-specific, the quality and utility of the test are largely determined by the reagents used and the subsequent steps in the test format. It has been rarely used except for an agglutination inhibition test for DEN (Chan *et al.*, 1975).

2. Neutralization Tests

The neutralization test (NT) measures all neutralizing immunoglobulins, including IgG and IgM (Ishii *et al.*, 1968). The excellent specificity for virus identification is well recognized. However, when used for serodiagnosis *in vitro*, its superior specificity primarily applies to the diagnosis of primary but not secondary infections. The three kinds of NT (constant virus–constant serum dilution, variable virus dilution–constant serum dilution, constant virus dilution–variable serum dilution) are generally performed *in vitro* using cell culture. The first test is useful when a large number of specimens must be processed economically, as in a serosurvey or when the neutralizing antibody (Nt) titer can be reasonably extrapolated based on plaque count at a fixed serum dilution (Sangkawibha *et al.*, 1984). The second test, assayed in laboratory animals, and all passive immunity tests using surrogate animals are useful for a small number of specimens, but are expensive, laborious, and impractical for processing of a large number of specimens.

The most popular method today is the third test, known as plaque-reduction serum dilution neutralization test (or PRNT) (Russell *et al.*, 1967a). To economize, laboratories most often perform microPRNT using multi-well plates (DeFraites *et al.*, 1999; Fujita *et al.*, 1975). Despite its importance in evaluation of vaccine efficacy and as a more

definitive, confirmatory test in serodiagnostics, neither the procedure nor diagnostic criteria have been standardized, given the numerous variations among laboratories, for example, in qualities of reagents, virus, cell culture, and protocols. Some of those problems and concerns are discussed in following sections.

Accurate determination of the proportion of a human population with a protective antibody is critical for planning a vaccination program. As described subsequently in Section V, development of flavivirus cross-reactive, heterologous antibodies demonstrating *in vitro* neutralization presents a serious problem. Currently, *in vitro* assays for Nt antibody often cannot adequately determine if heterologous, *in vitro* Nt antibodies are protective *in vivo* against the respective heterologous viruses based on the specimens demonstrating a pattern of secondary infection, when the history of flavivirus exposure of the subjects is poorly known.

a. Plaque Reduction Neutralization Test In this test, virus is pretitrated by plaque assay, and the heat-inactivated serum specimen is serially diluted. A known amount of infectious virus is mixed with an equal volume of each serum dilution and incubated. If the serum had a Nt antibody to the virus, reduction in the amount of infectious virus in the mixture occurs. The mixture is then inoculated into a susceptible cell monolayer and incubated for virus adsorption. After an incubation period, the monolayer is overlaid with a solid or semi-solid overlay medium. After an optimal incubation period, plaques are visualized with a dye. Using the mean plaque count of the virus dose mixed with an equal volume of a normal serum and applying a selected criterion for significant plaque reduction, the highest serum dilution demonstrating a significant plaque reduction is determined as the Nt titer. Alternatively, it is determined by probit analysis.

Basically, three kinds of overlay medium have been used: solid single overlay (Barnes and Rosen, 1974), solid double overlay (Russell *et al.*, 1967a; Yuill *et al.*, 1968), and semi-liquid overlay (De Madrid *et al.*, 1969). Although a particular overlay medium has been selected in most laboratories on the basis of personal preference or expertise available, generally, for the flaviviruses that grow more slowly (such as some strains of DEN-3 virus) a double overlay method is superior because an overlaid monolayer can be kept alive for several days or longer under the optimal condition favorable for plaque development, before a second solid medium containing a dye is applied for visualization of plaques.

As for virus, it is important to use a well-preserved stock because noninfectious virions react with Nt antibodies in the specimen, distorting the results (Schlesinger *et al.*, 1956). Usually, prototype viruses have been used. However, substantial differences in antigenicity exist among geographic strains of some flaviviruses, such as Southeast Asian vs pre-1990 Caribbean strains of DEN-3 virus (Russell and McCown, 1972), the Taiwan strains vs the Nakayama strain of JE virus (Susilowati *et al.*, 1981), and South African vs Indian strains of WN virus (Blackburn *et al.*, 1987). For specimens from those locations, use of local strains would provide more relevant data (Ku *et al.*, 1994).

The accuracy of plaque reduction first depends on the accuracy of plaque counts, in particular, the denominator, which is the virus dose that survives after being mixed with an equal volume of normal reference serum. The optimal range of the amount of virus for the plaque reduction neutralization test (PRNT) (plaque-forming units, or PFUs) depends on the mean diameter of the plaque of the virus strain used, the cell culture surface space available for plaque development per vessel, the susceptibility of the cell culture to plaque development, and the efficacy of the plaquing procedure. As demonstrated early, when vessels of a larger surface area are used, unless the plaque size is unusually large, a higher dose of virus can be inoculated without compromising the accuracy of the plaque count (Russell *et al.*, 1967a). However, in many laboratories, microPRNT with multi-well plates is used to process a large number of specimens economically. A proportional relationship between plaque count and serial dilution of virus exists only in a narrow range of virus dilution when a small surface area is used (Sukhavachana *et al.*, 1969). This is an important consideration for an accurate back titration. Thus, unless plaque size is very small, virus quantities much less than 50 PFUs have been found optimal for 24-well plates (Graham *et al.*, 1999), rather than nearly 100 PFUs (Lang *et al.*, 1999). Generally, whenever possible, large wells (i.e., 9.6 cm^2 of 6-well plates) or at least wells of intermediate size (4.5 cm^2 of 12-well plates) are much preferred. Despite its popularity, PRNT may not be the best NT for all flaviviruses, as reported for tick-borne encephalitis (TBE) virus (Vene *et al.*, 1998).

b. Focus, Cytopathic Effect, or Other Infectious Titer Reduction Tests As variants of PRNT, a few NTs were developed on the basis of reduction of infectious foci or cytopathic effects (CPEs) rather than of plaques. The principles of these tests are basically identical to those of PRNT, the only difference being the method of demonstrating

evidence of neutralization. Thus, in contrast to visual counting of plaques, infected foci or CPEs in cell culture that develop after inoculation of virus-serum mixture are counted microscopically (RFFIT and PAP) or macroscopically (CPE test). The same definition of Nt titer as that for PRNT is used in the former tests, while in the latter test, the highest serum dilution that inhibited CPE development is the Nt titer.

In the rapid fluorescent focus inhibition test (RFFIT) (Thacker *et al.*, 1978; Vene *et al.*, 1998) and peroxidase-anti-peroxidase (PAP) test (Ishimine *et al.*, 1987; Jirakanjanakit *et al.*, 1997; Okuno *et al.*, 1985), foci are counted with a fluorescence microscope or with a regular compound microscope. When results are compared with those obtained with PRNT, comparable results have been obtained by these focus reduction tests. Moreover, the RFFIT for TBE was reported to be faster and more reproducible than PRNT (Vene *et al.*, 1998). Two major disadvantages common to focus reduction methods are tedious, time-consuming counting of foci with a microscope and error in scoring by less experienced operators. The CPE reduction test suffers not only from subjectivity of scoring due to variation in the definition or visual perception of CPE among operators but also from variation in susceptibility of the cell culture used.

In another modification of the NT protocol, after a known amount of infectious virus was mixed with a serum specimen, the mixture was inoculated into a suspension of susceptible cells in the wells of multi-well tissue culture plates. Infectious virions that survived neutralization and replicated were titrated by ELISA to deduce Nt antibody titer in serum (Holzmann *et al.*, 1996; Vorndam and Beltran, 2002). Although they may be useful for primary infections, the frequent problems in all similar tests, including quantification of replicated virus with reverse-transcriptase polymerase chain reaction (Ting *et al.*, 2001), were the difficulty of regulating viral growth and establishing a reliable correlation between the amount of infectious virus and Nt antibody titer due to a rapid change of the slope of the relationship over a short period and of obtaining reproducible differences in titer in paired specimens, particularly in secondary infections.

c. Metabolic Inhibition Neutralization Test In this test, like PRNT, a known amount of infectious virus is added to each set of serial dilutions of serum. The diluent for both virus and serum dilutions is a metabolic medium containing a higher concentration of glucose. After incubation, first the serum-virus mixture and then an aliquot of cell suspension are dropped in each well of a microtiter plate. Cell controls

include serial twofold dilutions of cell suspension. Wells are then sealed and incubated at 37 °C for a desired period. Wells are scored Nt antibody–positive (lower pH) or Nt antibody–negative (higher pH) with respect to neutralization, using a pre-determined cut-off pH value in cell culture, such as 7.4. Adding the correct amount of cells and incubating for an optimal length of period are critical because adding too many cells accelerates metabolism and reduces the pH quickly, while the opposite occurs if too few cells are added. This test has been used for detecting antibodies to a few tick-borne viruses (louping ill and tick-borne encephalitis viruses) (Kääriäinen, 1965) and for serosurveys of SLE virus infection in wildlife in North America.

3. Enzyme Immunoassay

The application of enzyme immunoassays (EIAs), in particular, the ELISA that began in late 1960s, dramatically changed serologic practices by the late 1980s and spawned numerous procedural modifications and commercial diagnostic kits. Provided that basic equipment are available in all laboratories, the selection of a format depends on (i) the targeted molecules (antibody or antigen) for assay, (ii) availability of necessary reagents, (iii) the specificity and sensitivity desired, (iv) speed of test, and (v) expertise available or personal preference.

a. Types of Protocols Among many available protocols and modifications, the antibody capture format has been used for most flavivirus diagnoses. Before reaction with the specimen, a solid phase is sensitized either with virus antigen, anti-virus antibody, or anti-human IgG or IgM antibody. When the solid phase is sensitized with density gradient-purified DEN antigen and serial serum dilutions are used, the test is fast and the color pattern that develops by IgG-ELISA is very much similar to the HI pattern obtained (Fig. 1A) (Feinstein *et al.*, 1985; Kuno *et al.*, 1991). On the other hand, in other IgG capture ELISA protocols, the solid phase was sensitized with a polyclonal antiserum (hyperimmune mouse ascitic fluid or HIMAF) (Fig. 1B), which yielded good sensitivities (Chungue *et al.*, 1989; Miagostovich *et al.*, 1999). For IgG capture, the solid phase may be sensitized with anti-human IgG antibody (Burke *et al.*, 1985b; Innis *et al.*, 1989), but the sensitivity has been found to be generally inferior because a small amount of virus-specific IgG has to compete for binding sites with much higher concentrations of nonspecific IgGs. IgG-ELISA could be made more broadly reactive to a spectrum of flaviviruses, like the HI test, by sensitizing the solid phase with a *Flavivirus* group-reactive monoclonal antibody (MAb) (Johnson *et al.*, 2000).

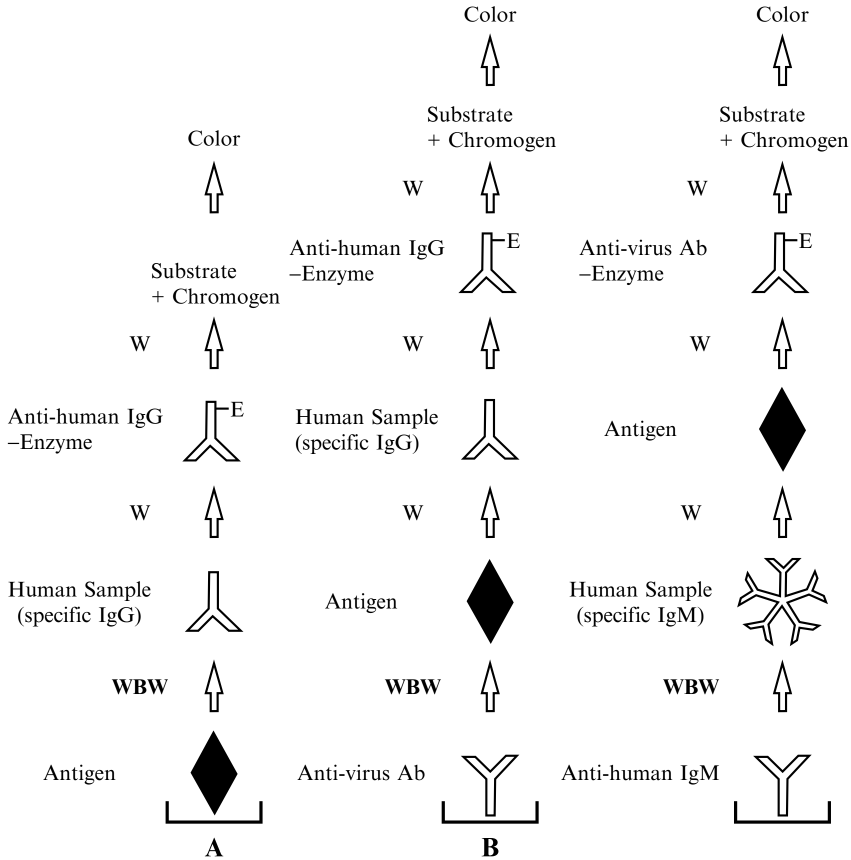

FIG 1. Examples showing variation of indirect ELISA format for antibody assay using horseradish peroxidase. (A) Antibody capture by a specific antigen. (B) Antibody capture by a specific antibody. (C) IgM capture by an antibody against human IgM. E, enzyme; WBW, washing-blocking-washing.

For investigating the immune responses among IgG subclasses, viral antigen-sensitized plates are reacted first with serum sample and then with mouse anti-human IgG1, 2, 3, or 4 antibody before the final reaction with an enzyme-conjugated antibody against mouse IgG (Thein *et al.*, 1993).

For IgM assay, although an antigen-sensitized solid phase could be used (Dittmar *et al.*, 1979), solid phase sensitized with anti-human IgM (Fig. 1C) (Burke and Nisalak, 1982; Gadkari *et al.*, 1984; Heinz

et al., 1981; Kuno *et al.*, 1987; Roggendorf *et al.*, 1981) has proven to be quite useful for nearly all medically important flaviviruses assayed thus far. As one other modification of ELISA, antigen and enzyme-conjugated detector antibody were incubated simultaneously to speed up the test apparently without compromising sensitivity (Chong *et al.*, 1994). Use of biotin-labeled anti-*Flavivirus* IgG, followed by streptavidin-peroxidase conjugate, was reported to have increased sensitivity of IgM-ELISA in acute phase specimens (Kittigul *et al.*, 1998).

i. Antigen or Immune Complex Capture ELISA Antigens in early acute phase specimens may be detected by antigen capture (AgC) ELISA. Most often specificity is enhanced by using a specific MAb as capture or detector antibody (Heinz *et al.*, 1986; Kuno *et al.*, 1985; Monath and Nystrom, 1984). ELISA procedures in which an anti-DEN NS1 polyclonal antibody was used as capture antibody revealed a higher level of NS1 in the acute phase of DEN infections (Alcon *et al.*, 2002; Young *et al.*, 2000).

A biotin-streptavidin amplification step was applied to improve sensitivity and specificity in another protocol (Malergue and Chungue, 1995).

DEN immune complex (IC) was investigated intensely with regard to the pathogenesis of DHF/DSS. The validity of early investigations was not entirely certain because the techniques used were not antigen-specific (Agnello, 1980). By a simple modification of IgM capture ELISA, the IgM IC of DEN virus or JE IgG- or IgM-IC also could be detected (Desai *et al.*, 1994; Kuno *et al.*, 1987).

ii. Blocking ELISA Because IgG ELISA is generally cross-reactive, to make the assay more virus-specific or strain-specific, modified, competitive protocols have been developed. In blocking ELISA, competition is allowed to proceed in sequence, first with test specimens, followed by the introduction of a competitive antibody without washing the plates. If optical density (OD) is significantly reduced as a result of blocking or inhibition, the presence of specific antibody in the samples is assumed. Thus, in such blocking tests, JE could be distinguished from DEN (Burke *et al.*, 1987) and Murray Valley encephalitis (MVE) from other Australian *Flavivirus* (Alfuy and Kunjin) infections (Hall *et al.*, 1995; Hawkes *et al.*, 1990). Inhibition ELISA is a simple modification of blocking ELISA developed for dengue diagnosis (Balmaseda *et al.*, 2003; Vázquez-Ramudo and Fernández-Lianes, 1989). In this test, the solid phase, which is coated with anti-DEN antibody, is first reacted with DEN antigen and then with a human serum dilution specimen. If the specimen had anti-DEN antibody, it will

coat the DEN antigen captured in the previous step, thus blocking ("inhibiting") it from binding an enzyme-conjugated anti-DEN human antibody to be introduced in a subsequent step. In contrast to blocking ELISA, however, the plates are washed between the serum specimen and enzyme conjugate steps. The highest serum dilution demonstrating $\geq$50% inhibition of absorbance (compared with that of a negative control serum) is used to determine antibody titer. *Flavivirus* cross-reactivity is a problem with this test.

b. Reagents and Procedural Modifications

i. Antigens and Enzyme-Conjugated Antibodies Sucrose-acetone extract of infected suckling mouse brain used to be the most common source of antigens for ELISA (Roggendorf *et al.*, 1981). Later, virus grown in cell culture became an important source of viral antigen (Besselaar *et al.*, 1989; Cardosa *et al.*, 1992). Also, for simplifying the assay of antibodies to DEN complex viruses, tetravalent antigen has been used. It has been recognized, however, that some specimens have positive results with monovalent antigen rather than with tetravalent antigen, and vice versa (Igarashi and Antonio, 1997). Recombinant antigens are now considered not only as a viable but necessary alternative to the mouse brain or cell culture antigens. Recombinant antigens, consisting of premembrane and envelope (E) proteins or E protein alone, have been found to be useful for diagnosis of DEN (Cuzzubbo *et al.*, 2001; Konishi and Fujii, 2002; Makino *et al.*, 1991), JE (Hunt *et al.*, 2001; Konishi *et al.*, 1996, 2001), TBE (Heinz *et al.*, 1995; Marx *et al.*, 2001; Yoshii *et al.*, 2003), and WN viruses (Davis *et al.*, 2001). Viral proteins expressed as fusion proteins of *Escherichia coli* for DEN viruses (Fonseca *et al.*, 1991; Makino *et al.*, 1991) are also useful, but their applications require special care because of the need to subtract the high background caused by the reactions of anti–*E. coli* protein antibodies present in nearly all human serum specimens and because of the lack of reaction in early specimens (Simmons *et al.*, 1998). Also, for vaccinia constructs, such as that for JE virus, minor contamination of vaccinia virus antigen in the recombinant antigen preparation affects the results of serum specimens from smallpox vaccinees (Konishi *et al.*, 1996).

Infected cells fixed on a solid phase are also useful in ELISA. Cell-associated antigen has been used in ELISA of DEN, WN, and YF viruses (Ansari *et al.*, 1993; Figueiredo and Shope, 1987; Soliman *et al.*, 1997). Although most antigens used in ELISA consist of envelope protein, DEN and JE virus NS1 antigens either affinity-purified from infected cell culture or expressed in eukaryotic cells by recombinant

plasmid were also found to be useful (Huang *et al.*, 2001; Konishi and Suzuki, 2002; Shu *et al.*, 2000).

Regarding the quality of antibodies, MAbs are more advantageous for reducing specificity variation than polyclonal antibodies. For enzyme-conjugated antibodies, broadly flavivirus cross-reactive MAbs, such as 4G2 (Gentry *et al.*, 1982) and 6B6C-1 (Roehrig, 1982), have been most popularly used as detector antibodies.

ii. Removal of IgG from Specimens To improve the specificity of IgM ELISA, removing IgG from specimens has often been recommended, particularly when samples contain too much specific IgG. In one study, the use of an anti-human IgG antibody was reported to have yielded improved results (Reinhardt *et al.*, 1998). However, in another study in which the efficacies of three IgG absorbents (RF adsorbent, protein G adsorbent, and *Streptococcus pyrogenes*) were evaluated, no improvement was observed for DEN (Kheong *et al.*, 1993). Similar negative results were also obtained using several commercial IgG adsorbents elsewhere (Kuno, unpublished).

iii. Reducing Background Sensitized plates are routinely blocked with a blocking material, such as non-fat dry milk, animal serum protein, or Tween 20, before the reaction with the specimen is begun. Diluting antigen and/or enzyme-conjugated detector antibody in acetone-extracted normal human serum (NHS) has been shown to greatly reduce background (Innis *et al.*, 1989).

4. Immunoblot and Immunochromatographic Tests

Instead of a multi-well plate used in ELISA, a membrane strip is used in a variation of the EIA as a dipstick or in an immunochromatographic assay. Strips may be held upright for vertical diffusion of reagents or horizontally as in cassette kits. A plain membrane strip may be used, but membrane impregnated with reactant(s), such as capture antibody, and pre-blocked is more useful for a rapid test. In some diagnostic kits for DEN, a recombinant envelope protein is used (Ludolfs *et al.*, 2002; Cuzzubbo *et al.*, 2001). Both IgM and IgG capture protocols have been developed (Cardosa and Tio, 1991; Cardosa *et al.*, 1995; Devine *et al.*, 1997; Wu *et al.*, 1997).

5. Immunofluorescent Antibody Test and Western Blot

Indirect immunofluorescent antibody tests (IFA) have been used for serodiagnosis of DEN (Boonpucknavig *et al.*, 1975), WNF (Besselaar *et al.*, 1989), and YF infections (Monath *et al.*, 1981; Niedrig *et al.*, 1999). Cross-reaction among flaviviruses was observed in a JE study (Yamagishi *et al.*, 1977). Two other problems with the method are that

it involves a tedious and time-consuming examination of fluorescence and that reading fluorescence accurately depends on the competence of the operator. Thus, a survey of the literature clearly shows that IFA has yielded more reports of false results than other major serologic methods. Also, an IgM assay with IFA is generally less specific than an IgM ELISA. Additionally, the residual infectivity of virus, such as SLE virus, in acetone-fixed slides is a biosafety concern (Yabrov *et al.*, 1978).

Western blot is useful for analyzing antibody responses to all viral proteins. One of the major interests of the use of Western blot for dengue has been to determine if it can distinguish dengue fever (DF) from dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS) (Churdboonchart *et al.*, 1990; Kuno *et al.*, 1990; Shu *et al.*, 2000; Valdes *et al.*, 2000). Also, it was reported that detection of antibodies to nonstructural proteins (NS) of DEN virus depended on the type of cell culture used for preparation of viral antigens (Se-Thoe *et al.*, 1999). Detection of the antibody to prM protein was reported to be useful for distinguishing DEN from JE and WNF (Cardosa *et al.*, 2002). When this technique was used for CSF specimens of JE, the antibody profile was found to be different from that of serum specimens (Patarapotikul *et al.*, 1993).

III. Specimens

A. *Specimen Collection*

1. *Source of Specimen and Timing of Collection*

Blood specimens constitute most diagnostic samples. Virus-specific IgM is detectable even in blood collected shortly after birth from infants congenitally infected with DEN virus (Boussemart *et al.*, 2001; Poli *et al.*, 1991). Cerebrospinal fluid (CSF) specimens from patients demonstrating central nervous system (CNS) syndrome are also sources of IgG, IgM, and IgA (Ehrenkranz *et al.*, 1974; Günther *et al.*, 1997; Han *et al.*, 1988). In JE, SLE, TBE, and WN, specific IgM is often detectable on admission when viral RNA in CSF and in blood may be no longer detectable (Ehrenkranz *et al.*, 1974; Fine *et al.*, 2000; Günther *et al.*, 1997; Morita and Igarashi, 1992).

As for IgM in blood, specimens collected too early (within a few days after onset) in nonneurotropic flaviviral infections, such as DEN, often do not demonstrate measurable titers, because IgM becomes detectable usually 3–10 days after onset, depending on virus and host.

In those cases, which tend to give false-negative results, most ideally, additional acute phase specimens need to be obtained. Blood specimens from patients who received blood transfusion within a few months before sampling should be examined carefully, since anti-flavivirus antibodies, such as anti-WN virus antibody, are sometimes detected in blood used for transfusion (Charrel *et al.*, 2001) and because the half-life of injected anti-flavivirus IgG was at least 26 days in adults (Adner *et al.*, 2001).

In addition, saliva in the acute phase has been found to be a source of specific IgM, IgG, and IgA in DEN (Artimos de Oliveira *et al.*, 1999; Balmaseda *et al.*, 2003; Cuzzubbo *et al.*, 1998).

2. Filter Paper

Most blood specimens are intravenously collected in tubes, but filter paper strips or discs also have been found useful to save money, to obtain blood from infants via finger or foot pricking, and to facilitate the shipment of samples through the postal service in a surveillance program over a large territory (Bond *et al.*, 1969; Burke *et al.*, 1985a; Sangkawibha *et al.*, 1984; Top *et al.*, 1975; Vázquez *et al.*, 1998).

B. Physicochemical Factors Adversely Affecting the Qualities of Specimens and Interfering Molecules

Heat inactivation at 56 °C for 30 minutes is a standard procedure in diagnostic laboratories to perform Nt or to prevent inadvertent laboratory infection with bloodborne agents. Although adverse effects on arboviral serology have not been widely recognized, elsewhere heat treatment has been identified as the cause of distorted results in serologic tests of other viruses, including HIV and arenavirus (CDC, 1989; Tomori *et al.*, 1987), and in the loss of Nt enhancing factors (Chappell *et al.*, 1971; Lehmann-Grube, 1978; Porterfield, 1980). Accordingly, in PRNT of flaviviral infections sometimes the reaction is supplemented with complement or normal serum to compensate for the loss of "accessory factor" caused by heat inactivation and improve sensitivity (but not specificity) (Halstead, 1974; Lang *et al.*, 1999; Study Group, 1961; Vaughn *et al.*, 1996; Westaway, 1965; Wisseman *et al.*, 1962). Conversely, storage at −20 °C for long periods or repeated cycles of freezing and thawing are known to reduce CF and Nt titers of many specimens (Goldblum *et al.*, 1957; Porterfield, 1980). The IgM titer in dried specimens on filter paper declines rapidly at any temperature, when kept for a long period (Cohen *et al.*, 1969), whereas the IgG decay in dried specimens is much slower when stored at 4 °C for 4–6 months (Chungue *et al.*,

1989; Cohen *et al.*, 1969). Accordingly, it was recommended that IgM test of filter paper specimens be performed within a relatively short period, such as 1 month after sample collection (Ruangturakit *et al.*, 1994; Vázquez *et al.*, 1998). However, the advice not to use diluted IgM-positive serum specimens kept at 4 °C for more than 10 days (Martin *et al.*, 2000) was not supported by others (Wong and Seligman, 2001). In the HI test, natural hemagglutinins are removed with erythrocytes and non-specific inhibitors with acetone or kaolin. Rheumatoid factor in serum was found to interfere with IgM ELISA in TBE and DEN diagnoses (Jelinek *et al.*, 2000; Roggendorf *et al.*, 1981).

IV. Variations in Antibody Responses and Antibody Kinetics

A. *Introduction*

During the early studies of HI and CF antibody responses to natural cases of YF, it became apparent that most immune responses could be classified into two patterns, primary and secondary infections (Theiler and Casals, 1958). This and other observations laid the foundation for serologic characterization of flaviviral infections. When analyzed for each immunoglobulin class, as described in the previous section, antibody response, in terms of temporal and quantitative dynamics, is different between different kinds of immunogen (i.e., wild versus vaccine strain), primary and secondary infections, or between sources of specimen, such as serum and CSF. Full understanding of human antibody responses to flaviviral infections is essential for a better serodiagnosis.

B. *Primary Infections*

1. IgG

In blood, the IgG titer begins to rise shortly after the IgM titer in the acute phase, but peak titers are generally lower than those in secondary infections. In many YF patients, the antibody begins to appear towards the end of the first week in the acute phase of illness, and the titer gradually rises thereafter (Lhuillier and Sarthou, 1983). The long-term persistence of Nt (and even CF) antibodies has been well recognized (Fujita and Yoshida, 1979; Halstead, 1974; Niedrig *et al.*, 1999; Poland *et al.*, 1981; Sawyer, 1931).

According to the classification of DEN, when paired specimens are collected more than 7 days apart and showing $\geq$ four-fold rise in HI titers, with the highest titer $\leq$1280, they are considered to be the cases

of primary infection (WHO, 1997). Whereas titers decline in the first several months in most patients, a high Nt titer is maintained for at least 2–5 years in some patients who have naturally been infected, in particular, with neurotropic flaviviruses, such as WN and JE viruses (Buescher *et al.*, 1959; Goldblum *et al.*, 1957).

In CSF samples of neurotropic flaviviral infections, IgG becomes detectable generally shortly after the IgM appearance and peaks later than IgM or IgA but may be detectable even 1 year after onset, as in louping ill (LI) and TBE (Günther *et al.*, 1997; Webb *et al.*, 1968) or well over 6 months in JE (Burke *et al.*, 1985b). Salivary IgG titer in DEN was reported to correlate with serum HI titer and was found useful in distinguishing primary from secondary DEN (Cuzzubbo *et al.*, 1998). A temporal increase in the avidity of IgG after primary infection was reported for TBE. Using a high avidity index after urea treatment as a marker, many cases that were clinically compatible but IgM-negative could be confirmed as current cases of TBE (Gassman and Bauer, 1997).

2. IgM

The IgM titer in blood in natural DEN begins to rise earlier, a few to several days after onset than HI or Nt antibodies, and by the tenth day of illness, more than 75% of the patients exhibit IgM (Nogueira *et al.*, 1992a). In YF, similar IgM positivity in 12 days has been observed among vaccinees (Reinhardt *et al.*, 1998); and in natural infections, the antibody titer rises sharply in the acute phase (Lhuillier and Sarthou, 1983). Development of envelope protein-specific IgM was also demonstrated in the recipients of a chimeric YF-based JE vaccine candidate (Monath *et al.*, 2002).

In infections by nonneurotropic flaviviruses, IgM reaches a peak in a few weeks, but then becomes undetectable generally between 1 and 3 months after illness onset. In some YF vaccinees, however, IgM in serum persisted for 18 months (Monath, 1971). Also, in an exceptional DEN case with a CNS syndrome, IgM was detectable longer than 250 days after onset (Chen *et al.*, 1991). Similarly, in a rare case of Edge Hill virus infection, IgM persisted for longer than 22 weeks (Aaskov *et al.*, 1993). In neurotropic viral infections (JE, SLE, TBE, WNF), however, its persistence in blood over 6 months or longer is not uncommon (Edelman *et al.*, 1976; Günther *et al.*, 1997; Han *et al.*, 1988; Kayser *et al.*, 1985; CDC, 2000). In contrast to HI or Nt titers, serum IgM titers in primary infections are higher than those in secondary infections (Innis *et al.*, 1989; Lhuillier and Sarthou, 1983).

In CSF, in the acute phase, generally the IgM concentration is higher than in blood and becomes detectable slightly earlier than IgG. In JE, many patients were IgM-positive after 6 months (Burke *et al.*, 1985b), and nearly 33% of the patients with TBE demonstrated IgM 1 year after the onset of illness (Günther *et al.*, 1997). On the other hand, in DEN infection presenting with a CNS syndrome, IgM persisted for less than 1 month (Chen *et al.*, 1991; Lum *et al.*, 1996). Accordingly, coupled with the aforementioned persistence of specific IgG, the standardized application of IgM detection within 45 days after onset of illness as a collateral evidence of recent infection (Martin *et al.*, 2000) is sometimes difficult for the diagnosis of patients with a history of infection (clinical or subclinical) by a neurotropic flavivirus within a year or so earlier.

In saliva, anti-DEN IgM was detectable in 82% of specimens collected $\geq$5 days after onset (Artimos de Oliveira *et al.*, 1999), and titers were higher in primary than in secondary infections (Cuzzubbo *et al.*, 1998).

3. IgA

IgA is the most abundant antibody in secretions and is the first line of defense for the viruses that gain entry by direct transmission. However, since under normal circumstances few flaviviral infections are transmitted to human by this mode, the protective significance of this antibody in secretions is not clear.

In a trial of a YF virus vaccine, the profile of IgA response in blood paralleled that of IgG, but its titer became undetectable after 62 to 82 days (Monath, 1971). In TBE, the titer peaked in 2 months (Günther *et al.*, 1997). In natural DEN, 17% of acute phase specimens and 100% of convalescent phase specimens were IgA-positive; and it became undetectable between 2 and 4 months after onset of illness (Groen *et al.*, 1999). This contrasted to the much lower proportions and shorter half-life reported earlier (Summers *et al.*, 1984). In other observations, the anti-DEN IgA titer in serum appeared about 1 day later than IgM, peaked around 8 days after onset, and became undetectable earlier than did IgM; and no difference in titer was found between primary and secondary DEN infections (Talarmin *et al.*, 1998).

In CSF specimens of patients with JE and TBE, IgA was detectable up to 6 weeks after onset (Günther *et al.*, 1997; Han *et al.*, 1988). IgA in the saliva of DEN patients had a half-life shorter than that of IgM (Cuzzubbo *et al.*, 1998). At least as far as IgA kinetics in serum is concerned, unlike IgA in secretions, because of the greater individual variation, generally, it has less diagnostic value than IgG or IgM.

4. Anti-NS1 Antibody

The NS1 antibody of DEN is infrequently detected by Western blot in primary infections, except in the late convalescent stage (Hoke *et al.*, 1990; Kuno *et al.*, 1990; Shu *et al.*, 2000). Recently, however, using a larger amount of a purified NS1 antigen made affordable by a recombinant technology, this antibody could be detected with ELISA in 88% of primary DEN infections (Huang *et al.*, 2001). A more recent study revealed a high proportion of patients (80%) positive for NS1 as early as 1 day after onset of illness in the absence of viral RNA or specific IgM, suggesting a potential use for early dengue diagnosis (Alcon *et al.*, 2002). Antibodies to NS proteins are induced only by infectious viruses with functional NS genes. Thus, the absence of anti-NS1 antibody among vaccinees of an inactivated JE vaccine could be used as a marker to differentiate serum specimens of JE vaccinees from those of natural infections (Shu *et al.*, 2001).

C. Secondary Infections

In terms of antibody responses, secondary flaviviral infections include natural sequential infections or vaccinations with two different flaviviruses, revaccination or booster immunization with the homologous virus, and a combination of a natural exposure to a flavivirus and a vaccination with another virus at different times.

1. IgG

Generally, an IgG titer in blood is often detectable in the early acute phase specimens, rises sharply and persists much longer than in primary infections. Higher Nt titer after secondary infection is observed after an exposure not only to complete virion but also to subunit viral immunogen as in a live, chimeric YF vaccine whose envelope gene was replaced with that of JE virus (Monath *et al.*, 2002). In DEN, HI titers $\geq$2560 on single specimens automatically classify the cases as secondary infections (WHO, 1997). Anti-DEN IgG titers in saliva were similarly higher in secondary than in primary infections (Cuzzubbo *et al.*, 1998).

In contrast to the aforementioned rapid rise in Nt IgG, in other individuals, the antibody response may be slower. For example, in 19 volunteers (15 of whom had received a JE vaccination 7–40 years earlier but no YF vaccine), anti-YF Nt antibody was detectable in 30% and 100% of the vaccinees on the tenth and 14th day after YF vaccination, respectively (Taga *et al.*, 2002). This prompted the investigators to propose a revision of the waiting period before validation, because

the current international certificate of YF vaccination becomes valid only 10 days after vaccination (CDC, 1999).

In DEN, the correlation of the change in IgG avidity with the development of DHF/DSS in secondary DEN has been the subject of research interest. In one IgG ELISA study using antigen-sensitized solid phase and twofold serial dilutions of serum (Kuno *et al.*, 1991), resistance to 6*M* urea treatment was evaluated in convalescent phase serum specimens (HI titer $\geq$ 160) from 10 patients with primary infection and 10 patients with secondary DEN infections who had much higher titers. The urea treatment reduced IgG titer fourfold to eightfold almost uniformly in both types of infections. Thus, the higher residual titers in secondary than in primary infections again reflected the differences in the original HI titers. Accordingly, the result did not reveal a disproportionately higher resistance to urea as expected if avidity of IgG actually increased in secondary infections (Kuno, unpublished).

2. IgM

The IgM response in secondary infections is generally slower and persists for a shorter period than in primary infections, as in DEN and YF, and the IgM titers are lower (Innis *et al.*, 1989; Lhuillier and Sarthou, 1983). Furthermore, in DEN, the proportion of individuals without an IgM response increases (Kuno *et al.*, 1991; Ruechusatsawat *et al.*, 1994). Nevertheless, in the YF vaccination trial of 19 volunteers described previously, all demonstrated anti-YF IgM on the 14th day after vaccination.

3. IgA

In a report of DEN, the proportions of serum specimens positive for IgA in acute and convalescent phases were 69% and 97%, respectively, and were comparable to IgM ELISA results (Groen *et al.*, 1999). In another report, anti-DEN IgA titers in the acute phase in patients with DSS were found to be significantly higher than those in DF patients (Koraka *et al.*, 2001).

4. IgE

The role of IgE in DHF pathogenesis was speculated on early, as hypersensitivity was considered one of the possible mechanisms of DHF/DSS. It was reported that IgE levels in serum specimens of DHF/DSS patients were significantly higher than those of dengue fever patients (Koraka *et al.*, 2003; Pavri *et al.*, 1979). In JE, IgE was detectable in acute phase serum but declined by the early convalescent phase (Pavri *et al.*, 1980; Shaikh *et al.*, 1983).

5. *Anti-NS1 Antibody and NS1*

This antibody could be detected in 100% of secondary DEN infections, using a sensitive ELISA (Huang *et al.*, 2001). Detection of NS1 or anti-NS1 antibody, however, could not be used as a marker to distinguish DHF/DSS from DF in secondary infections (Kuno *et al.*, 1990; Shu *et al.*, 2000) or primary from secondary infections (Alcon *et al.*, 2002).

D. *Tertiary Infections*

In locations endemic for several flaviviruses, tertiary (and quaternary) flaviviral infections occur (Myers and Varkey, 1971). In prospective studies of children ($\leq$10 years old) in Thailand and in Indonesia, 13–15% of children developed a tertiary infection (Graham *et al.*, 1999; Sangkawibha *et al.*, 1984). Also, the cause of the second episode of DHF/DSS experienced by some patients is speculated to be the result of third or fourth dengue infections (Nimmannitya *et al.*, 1990). Regarding its confirmation, however, a serologic response unique to tertiary infection has never been found; and a serologic classification of DEN as secondary or tertiary based on the number of DEN serotypes a serum (obtained prior to the latest DEN infection) neutralizes *in vitro* (Graham *et al.*, 1999) is, as described in the following sections (atypical "primary" infections and flavivirus cross reactivity), not supported by frequent observations of the development of cross-reactive Nt antibodies to variable number of serotypes after primary infections. Thus, many cases of tertiary (and quaternary) flaviviral infections have been most likely classified as cases of secondary infection when exact histories of flavivirus exposure were uncertain. In one of the tertiary infections with known medical history, a person was first infected with JE virus, then immunized with YF (17D) vaccine, and later challenged with DEN-1 virus. The highest Nt titers in blood collected after each exposure corresponded to JE, YF, and WN viruses, in that order (Wisseman *et al.*, 1966). In another individual who was first infected with DEN-4 virus, then immunized with YF (17D) vaccine, and lastly challenged with DEN-2 virus, the highest Nt titer after the third flavivirus exposure corresponded to DEN-2 virus (Bancroft *et al.*, 1981).

However, this type of matching between the virus involved most recently in a sequential flaviviral infection and the virus to which there is the highest antibody titer is not always observed, as in a case of tertiary infection in a YF vaccinee who later acquired natural DEN 4 and DEN 1 infections in that order (Carey *et al.*, 1965). Another person

who had a history of two YF vaccinations followed by DEN-1 infection received a three-dose TBE vaccination. An original antigenic sin phenomenon was observed, and anti-TBE–specific Nt antibody did not develop until after the third dose (Holzmann *et al.*, 1996).

E. Mixed Infections

Simultaneous exposure to more than one flavivirus antigen occurs when a person is bitten by a vector carrying two or more flaviviruses (Gubler *et al.*, 1985; Laille *et al.*, 1991; Myers and Carey, 1967) or immunized with a cocktail of viral vaccines. In two volunteers who were vaccinated with a mixture of attenuated DEN-1, DEN-2, and YF vaccines, the highest Nt titer corresponded almost monotypically to DEN-2 virus (Schlesinger *et al.*, 1956). On the other hand, in the volunteers immunized simultaneously with YF (17D) and a DEN-1 strain, Nt antibody titers to both viruses were similar (Fujita *et al.*, 1969). In four cases of natural bivalent infection with DEN-2 and DEN-4 viruses, the elevated CF titers to DEN-2 and DEN-4 viruses showed less than a twofold difference, but, in one patient, the DEN-2 titer was fourfold higher than the DEN-4 titer (Liao *et al.*, 1996). When a bivalent (DEN-2 and DEN-4) vaccine was inoculated into six volunteers, all developed Nt titers to the two serotypes (Bhamarapravati and Yoksan, 1989). However, with a tetravalent DEN vaccine, the highest Nt titer corresponded to DEN-3 virus in all volunteers (Kanesa-thasan *et al.*, 2001; Sabchareon *et al.*, 2002). The low proportions of the vaccinees who developed antibodies to four serotypes observed in those trials could be improved to about 70% in the latter trial by performing a booster immunization of the tetravalent vaccine formulation. In dual arboviral infections with a flavivirus and an alphavirus, $\geq$ fourfold rise in titer or very high IgM OD value has been found for both viruses (Myers and Carey, 1967; Thein *et al.*, 1992). Those examples clearly demonstrate the difficulty of predicting serologic outcomes of mixed flaviviral infections and of distinguishing mixed infections from sequential (secondary, tertiary, quaternary) infections without corroborating information on medical history. The process leading to final diagnosis of concurrent infections by a flavivirus and other arbovirus or non-arboviral pathogens (Amosov *et al.*, 2000; Lotric-Furlan *et al.*, 2001; Meehan *et al.*, 2000; Oksi *et al.*, 1993; Pancharoen and Thisyakorn, 1998; Phillips *et al.*, 1966; Sudjana and Jusuf, 1998) has been even more complicated and has not always been based on serologic evidence alone because of detection of both pathogens from

the same patients, of the similarity of clinical syndromes, and of other considerations by physicians.

F. Recurrent or Prolonged Infections

In three children who suffered recurrent infection with JEV, two demonstrated specific IgM in blood and CSF, and all three had a ≥ fourfold rise in HI titer 8 months after the first episode (Sharma *et al.*, 1991). On the other hand, in four of six cases of biphasic infections with JEV, a ≥fourfold increase in HI titer was not observed during the relapse that began 12–32 days after the first phase (Pradhan *et al.*, 2001). In a study of prolonged infection with JEV, IgM remained detectable in CSF for more than 1 year in three patients but was undetectable in three others, despite repeated viral isolation up to 117 days after infection (Ravi *et al.*, 1993). Far more serious examples of prolonged infections have been reported in TBE patients: a patient from whom a strain of virus was isolated during recurrent episode 17 years after onset (Pogodina *et al.*, 1981; Rubin and Chumakov, 1980); a patient who developed sudden increase in anti-RSSEV IgG and IgA titers in CSF during the relapse 13 years after initial infection (Ogawa *et al.*, 1973). In other cases, persistence of IgM was found intermittently in recurrent cases of TBE; nevertheless, the antibody still could be detectable in four patients 3 years after infection (Nadezhina, 2001). In a few patients with MVE, a sudden surge of HI antibody more than 5 months after onset was observed, including one individual who experienced the surge more than 15 months after a seemingly complete recovery, suggesting possible cases of prolonged infection (Doherty *et al.*, 1976).

G. Persistence of Antibody

A recent review revealed many examples of the lengthy persistence of IgG and IgM after flaviviral infections (Kuno, 2001). In the patients demonstrating a CNS syndrome of unknown origin, who had been vaccinated earlier with a TBE vaccine within 2 years before the current episode, the persistence of anti-TBE IgM in CSF posed not only a diagnostic complication but also a question of vaccine efficacy (Günther *et al.*, 1997). The occurrence of JEV IgM in CSF for more than 1 year and WNV IgM in serum over 500 days after onset were also reported (Ravi *et al.*, 1993; Roehrig *et al.*, 2003). In any locations where more than a few flaviviruses are active all year around (Talarmin *et al.*, 1998) or where repeated introduction is possible, lengthy IgM persistence renders correct etiologic identification more difficult. Similar

complications exist for determining an attack rate during or after an epidemic or for any seroprevalence studies.

H. Lack of Antibody Response

In vaccine efficacy trials, the lack of seroconversion in many vaccinees even after a booster injection (Craig *et al.*, 1999; Poland *et al.*, 1990; Susilowati *et al.*, 1981) has been partly attributable to the reduced immunogenicity of those vaccines, to immunologic interference, or to individual variation in immune response. Thus, after immunization with a JEV vaccine (SA-14-14-2), only 13% of the children demonstrated IgM 4 weeks later (Young *et al.*, 1999). Likewise, in a DEN-1 vaccine trial, eight volunteers developed Nt IgG but no IgM; conversely, three vaccinees developed IgM but no IgG (Edelman *et al.*, 1994). The lack of antibody response to a particular immunogen (or immunogens) of multivalent vaccines, as described earlier in mixed infections, most likely results from interference.

The absence of CF antibody in some naturally infected or vaccinated individuals was described earlier. In a study of anti-TBE antibody responses, HI and CF antibodies were negative in several patients, although specific IgM was detectable (Granström *et al.*, 1978). On the other hand, a considerable number of TBE patients demonstrated IgG but not IgM (Gassmann and Bauer, 1997). Similarly, in a report of Ilhéus virus (ILH) infection, no NT, CF, or HI antibodies could be demonstrated in five virologically confirmed patients, even 1 year after onset of illness (Nassar *et al.*, 1997). In another study, in two virologically confirmed cases of DHF, antibody to DEN serotypes did not increase significantly ($\geq$fourfold) (Makino *et al.*, 1994). Also, as mentioned earlier, the proportion of patients without IgM antibody in secondary DEN was higher than that in primary DEN (Kuno *et al.*, 1991; Ruechusatsawat *et al.*, 1994). A lack of IgM response was also reported in some YF patients (Monath *et al.*, 1981).

I. Physiologically or Immunologically Impaired Individuals

Nutritional deficiency has been reported to contribute to the lack of antibody response after YF vaccination (Brown and Katz, 1966) and in three of six cases of biphasic JEV infection (Pradhan *et al.*, 2001). A change in immunocompetence during pregnancy was suspected to be the cause of a lower rate of seroconversion after YF vaccination (Nasidi *et al.*, 1993), but in pregnant women infected with JEV, no immunologic impairment was reported (Chaturvedi *et al.*, 1980). The reports

showing a low or absent immune response in elderly JE and TBE vaccinees (Kusaba *et al.*, 1974; Marx *et al.*, 2001) have been also contrasted to other studies in which no significant reduction in immune response was found in the elderly group (Kanamitsu *et al.*, 1970).

In patients infected with human immunodeficiency virus (HIV), antibody responses to JE and YF vaccines were significantly lower than in healthy individuals (Rojanasuphot *et al.*, 1998; Sibailly *et al.*, 1997), and symptomatic SLE virus infection developed more often among patients with HIV (Okhuysen *et al.*, 1993; Wasay *et al.*, 2000). Similarly, in HIV-infected persons with hemophilia, the immune response to TBE vaccine was weaker than that in healthy persons (Panasiuk *et al.*, 2003; Wolf *et al.*, 1992). On the other hand, in other reports of YF or TBE vaccination, the immune responses of HIV patients were normal (Receveur *et al.*, 2000).

J. Atypical "Primary" Infections

Primary infections resulting in antibody responses compatible with those of secondary infections have occasionally been reported in individuals without previous flavivirus infection or vaccination (Barnes and Rosen, 1974; Graham *et al.*, 1999; Ishii *et al.*, 1968; Kanesa-thasan *et al.*, 2001; Vaughn *et al.*, 1996). Although the exact cause is not entirely clear, the three most likely explanations are (i) a previously unrecognized exposure to a flavivirus with the subsequent disappearance of detectable antibody, (ii) broadening of antibody specificity, and (iii) an atypical immune response by the host.

V. Flavivirus Cross-Reactivity

A. Introduction

The complex combination of cross-reactivity of antibodies, including their temporal and qualitative changes, of immunogens (or antigens used in diagnostic tests), and of the sequence of viruses involved in sequential infections profoundly complicates the interpretation of serologic results. For example, a fusion protein expressing the B domain of the DEN envelope protein did not cross-react with anti-YF or anti-JE IgG antisera, whereas cell culture–derived antigen did (Simmons *et al.*, 1998). In another report, cross-reactivity among DEN-2, WN, and YF viruses was, in turn, reported to be much lower with cell-associated antigens than with antigens prepared in cell culture

(Soliman *et al.*, 1997). Furthermore, when the roles of antigen and antibody were reversed between viruses evaluated for cross-reactivity, the results were not necessarily the same.

B. Primary Infections

1. IgG

The monotypic specificity of IgG in primary infections was recognized early. Even 30–48 years after patients were infected with DEN, the highest Nt antibody corresponded to either DEN-1 or DEN-4 virus, retrospectively identified as the original etiologic agents (Fujita and Yoshida, 1979; Halstead, 1974). Nevertheless, it was demonstrated in WNV fever that, although Nt antibody to the homologous virus was initially monotypic, after several months heterologous titers to JE virus and to a lesser extent to SLE virus appeared (Goldblum *et al.*, 1957). Similar observations were made in YF cases by NT assay (Theiler and Casals, 1958) and by IgG-ELISA in which cross-reaction of anti-YF antibodies with JE and WN antigens increased in the late convalescent stage, although homologous titers were still much higher (Ansari *et al.*, 1993).

Apart from the temporal increase in cross-reactivity, it has been often observed that when serum specimens were evaluated with antigenically closely related members within an antigenic complex (such as TBE, JE, and DEN complexes), even in the early convalescent phase of primary infections they were found to be more cross-reactive (Burke *et al.*, 1983; Hammon *et al.*, 1956; Price *et al.*, 1970; Russell *et al.*, 1967b; Vaughn *et al.*, 1996). Because IgG-ELISA is recognized to be cross-reactive even among the viruses belonging to more remotely related antigenic groups, such as Kunjin (KUN), JE, DEN-2, SLE, TBE, and/or YF viruses (Guo and Ryan, 1989; Tardei *et al.*, 2000), its application to etiologic agent identification is generally limited in flavivirus-endemic locations. In contrast to the considerable cross reaction common in most IgG-ELISA protocols, an ELISA for anti-NS1 antibody was reported to be DEN serotype-specific in primary infections (Shu *et al.*, 2002).

2. IgM

Early studies determined that IgM was more virus-specific than IgG (Westaway *et al.*, 1975). This was confirmed in studies of virus infections of major medical importance, such as DEN (Burke, 1983; Scott *et al.*, 1972), JEV (Edelman and Pariyanonda, 1973), MVE

(Wiemers and Stallman, 1975), Rocio (Iversson *et al.*, 1992), WNV (Tardei *et al.*, 2000), and YF (Lhuillier and Sarthou, 1983; Nogueira, 1992b). In most of these studies, however, cross-reactivity was evaluated primarily by using members of more antigenically distant groups. When it was evaluated among members of an antigenic complex, it was evident, for example, in approximately 15% of primary DEN-4 infections (Chungue *et al.*, 1989). Also, among 14 confirmed SLE patients, when IgM was reacted with five antigens (JE, MVE, SLE, WN, and YF), in three patients the IgM broadly reacted to all antigens (Monath *et al.*, 1984). Similarly, six of 32 serum specimens of confirmed SLE infections demonstrated higher P/N OD ratios to WNV than to homologous viral antigen (Martin *et al.*, 2002). Also, in 22% of 46 pediatric JEV patients, IgM cross-reacted with WNV antigen (Lowry *et al.*, 1998).

3. IgA and anti-NS1 Antibodies

In DEN patients and vaccinees, IgA was found to be cross-reactive to all four serotypes (Inouye *et al.*, 1980), but did not cross-react with YF virus (Groen *et al.*, 1999). Anti-DEN NS1 antibodies (IgG, IgM, and IgA) generally reacted monotypically among serotypes in 80%, 67%, and 75% of the specimens, respectively, and they did not cross-react with JEV NS1 (Shu *et al.*, 2001); however, cross-reactivity of anti-DEN NS1 IgM with JEV NS1 was reported by others (Huang *et al.*, 2001).

C. Secondary Infections and Original Antigenic Sin

1. IgG

In the classic study by Theiler and Casals (1958), the complexity of etiologic identification based on serologic results of secondary infections by YF virus was recognized. In another study, increased cross-reactivity among four DEN serotypes in the specimens of patients with secondary DEN was evident (Russell *et al.*, 1967b). By using immunoglobulin fractions obtained by density gradient centrifugation, IgG was identified as the source of much of the cross-reactivity (Makino *et al.*, 1994).

To identify the first or second etiologic agents involved in secondary infections, the concept of "original antigenic sin" has been sometimes applied, including investigations to determine the involvement of KUN rather than MVE virus among neurologic cases and to explain the occurrence of serum specimens with the highest Nt titer to DEN-3 in parts of Australia (Doherty *et al.*, 1967). The aforementioned antibody response

after a TBE vaccination (Holzmann *et al.*, 1996) is another example. In a study of eight children who suffered secondary DHF/DSS, the highest PRNT titers in convalescent phase specimens corresponded to the DEN serotypes involved in primary infections (Halstead *et al.*, 1983). In another study, however, three of nine specimens did not correspond (Kuno *et al.*, 1993). When persons first infected or immunized with JE vaccine were later infected with DEN-4 virus or vaccinated with YF vaccine, the highest Nt titers corresponded instead to DEN serotypes and to YF virus, respectively (Okuno *et al.*, 1982; Wisseman *et al.*, 1962). Also, when individuals with a history of YF vaccination were later vaccinated with DEN-2 or DEN-3 vaccine, the highest Nt titer corresponded to DEN-2 virus in four and DEN-3 virus in two individuals, respectively (Bancroft *et al.*, 1981; Kuno *et al.*, 1993). Available data regarding antibody specificity in multiple specimens obtained over a longer period after secondary infection (Burke *et al.*, 1987; Halstead *et al.*, 1983; Kuno *et al.*, 1993; Makino *et al.*, 1994; Okuno *et al.*, 1982; Schlesinger *et al.*, 1956; Wisseman *et al.*, 1962) reveal that the period of dominant antibody specificity to the first antigen after secondary infection lasted for a highly variable period ranging from less than a week to sometimes more than several weeks, at the end of which the dominant antibody specificity switched to that of second virus. It is not clear how long the specificity of the second virus dominates because blood specimens have been rarely obtained consecutively 6–8 months after the onset of illness. Most convalescent phase specimens are collected within 2–8 weeks after onset, a period when specificity switch may or may not have occurred, hence the source of the conflicting results. This may explain why late convalescent phase specimens were more useful in blocking ELISA for etiologic identification of the second virus (Burke *et al.*, 1987) and why increasing IgG immediately after the first dose of TBE vaccine did not have any Nt antibody to TBE virus in those vaccinees with a history of YF vaccination (Holzmann *et al.*, 1996).

Despite the popular application in serodiagnosis and the occurrence of compatible cases of human flaviviral infections, as well as laboratory evidence typically obtained in a murine model to support this phenomenon, there have been conflicting serologic results reported in numerous cases of other human viral infections as well as in animal experiments, besides the examples cited here (Williams *et al.*, 2001).

Furthermore, the number of cases of secondary flavivirus infections chronologically studied for a shift in serologic specificity over many months is still very small. Thus, the generalized application of this

phenomenon for the diagnosis of secondary flaviviral infections with uncertain medical history is risky.

2. *IgM, IgA, and anti-NS1 Antibodies*

In secondary infections, the IgM fraction contains antibodies specific to each of the two viruses involved (Scott *et al.*, 1972). Thus, like IgG, IgM in secondary infections is generally cross-reactive. As expected, in secondary DEN infections, the correct serotypes were identified by the highest IgM titer in only nine of 16 cases (Burke, 1983). In volunteers with a history of YF vaccination, after DEN-2 or DEN-1 vaccination, the highest IgM titers corresponded to DEN-2 and DEN-1 viruses, respectively (Figueiredo *et al.*, 1987; Scott *et al.*, 1983); and after JEV, in one patient it corresponded to JEV and in two patients, it corresponded to YF virus (Makino *et al.*, 1994). Also, when an OD IgM/OD IgG ratio was used to segregate DEN from JEV infections, the optimal cut-off ratio had to be raised from 1.79 in primary infections to 3.16 in secondary infections (Innis *et al.*, 1989). Anti-DEN NS1 antibody was found to cross-react with JE, SLE, and YF antigens (Kuno *et al.*, 1990). Similarly, the specificities of anti JEV NS1 IgG, IgM, and IgA were only 50%, 22%, and 30%, respectively (Shu *et al.*, 2000).

VI. Properties of Serologic Tests

A. Diagnostic Criteria

In each serologic test, a criterion (or criteria) is established for discriminating positive from negative specimens. Despite the importance of those criteria, insufficient attention has been paid to the adequacy of diagnostic criteria that are used routinely.

1. *Fold Difference in Titer in Paired Specimens*

In the serodiagnoses of infectious diseases in general, ≥fourfold difference (increase or decrease) in titer in timely, paired specimens has been one of the most popular criteria. This approach has been adopted for the classification of DEN by the World Health Organization (WHO, 1997), and similar sets of criteria have been established internally in some laboratories for the diagnoses of infections by other flaviviruses, such as SLE (Calisher and Poland, 1980). However, confirmed cases that did not meet the criteria have been reported. For example, in a study of 15 virologically confirmed DHF patients, a ≥fourfold rise in Nt titer was observed to both DEN and JE viruses and, in another case, only to JE virus (Makino *et al.*, 1994).

According to the WHO criteria (WHO, 1997), a ≤twofold difference in HI titer between specimens collected ≥7 days apart is not considered evidence of current DEN infection. However, cases that fell into this category but that were confirmed as recent cases by a rising IgM titer, a ≥fourfold rise in CF antibody or virus isolation, have not been uncommon in flavivirus infections (Bancroft *et al.*, 1981; Kuno *et al.*, 1991; Makino *et al.*, 1994; Wiemers and Stallman, 1975). It was also determined in an investigation of a JEV outbreak that 15–20% of the patients demonstrated no increase in HI titer (Burke *et al.*, 1985a). Similarly, in several patients presenting in the second phase of recurrent JEV infection, a ≥fourfold change in antibody titer was not observed (Pradhan *et al.*, 2001). It is also emphasized that the significance of fourfold differences at high and low antibody concentrations are not the same when sample dilutions form an exponential series of 2^n (Griffiths, 2001).

2. Plaque or Focus Reduction End Point

In most reduction tests, the lowest serum dilution tested is 1:10, unless there exists a compelling reason to test at 1:5. Unlike HI or CF tests, for PRNT, in the absence of a universally acceptable guideline, a variety of plaque reduction criteria, ranging from 50% to as much as 100%, have been used. High reduction criteria (i.e., 90%) have been often used to absolutely ensure the presence of Nt antibody. Regarding the scientific basis for adopting a particular criterion, in contrast to the plaque reduction criteria of 50% and 70% that were determined by well-designed studies (Morens *et al.*, 1985; Russell *et al.*, 1967a), the data validating the adequacy of 80% and 90% reduction criterion for flaviviruses (Barnes and Rosen, 1974; Calisher and Poland, 1980) were either unclear or based on the correlation with a neutralizing index (NI) obtained earlier (Earley *et al.*, 1967). However, NI obtained in animals has been recognized to be more variable and insensitive, requiring 1.7 log or greater for the difference in titer between paired specimens to be significant (Hammon and Sather, 1969; Porterfield, 1980; Schlesinger *et al.*, 1956). The higher reduction criteria deserve a careful evaluation because of their popularity, particularly in microPRNT. When a criterion with a high level of reduction, such as ≥90% is used, due to chance variation, the test becomes less sensitive to a small but significant (i.e., fourfold) difference in titer (Mannen *et al.*, 1987). Greater variation in titer becomes evident when development of a few plaques, which is 10% of virus dose (usually much less than 50 PFUs used in most micro tests), differentiates positive from negative specimens (Sukhavachana *et al.*, 1969). Moreover, even when

50% reduction criterion was used, Nt titers obtained were lower with multi-well plates than those determined by macroPRNT with 1-oz bottles (Jirankanjanakit *et al.*, 1997). A comparative study of JE Nt antibodies among three institutions using different criteria (50%, 80%, and 90%) demonstrated that when Nt titers were high, as in secondary infections including booster vaccinations, no significant difference in result was apparent among the three reduction criteria; but the titers obtained with 90% reduction criterion were five- to 19-fold lower than the others (DeFraites *et al.*, 1999). As expected, when Nt titers were low, with a 90% reduction criterion, more false-negative results were obtained than with lower reduction criteria, as demonstrated in a comparative study for DEN antibodies (Morens *et al.*, 1985). On the other hand, a 50% reduction criterion produces occasional false-positive results (Morens *et al.*, 1985), although it could be alternatively interpreted to suggest a higher sensitivity. Thus, a conservative 70% reduction criterion with larger wells (preferably in six-well plates but at least in 12-well plates, rather than in 24- or 96-well plates) is more desirable. At any rate, in the absence of a gold standard, in each laboratory a reliable criterion must be firmly established; when multiple well plates (such as 24-well and 96-well) are used with a 90% reduction criterion, it is desirable to take into consideration a possible increase in false-negative results on the specimens with low Nt titers before interpreting the results, and to consider supplementing the reaction with normal serum to enhance neutralization, although PRNT is not necessarily improved for all arboviruses with this supplement.

3. Criteria Used in ELISA

No serologic technique shows more variation than ELISA in terms of serum dilutions selected, reagents used, and diagnostic criteria adopted.

a. Optical Density (OD) and OD Ratio Basically, two kinds of cutoff values have been used. The first is a mean of the OD values of negative control wells plus a multiple (usually two or three times) of the standard deviation (SD). The second is a ratio of the OD of a positive control (P) sample to the OD of a negative control (N) sample (Monath *et al.*, 1984). In another modification of this method, the ratio of OD values obtained for IgM and for IgG was used to segregate primary from secondary DEN infections and the IgM OD ratio between DEN and JEV for ruling out the latter (Vaughn *et al.*, 1997). Whichever type of criterion is selected, a good compromise is generally necessary,

because false-positive (type I error) and false-negative (type II error) results are encountered whenever a large number of specimens are tested. Moving the cut-off OD or OD ratio to a higher value may minimize false-positive results, but likely at the expense of false-negative results (or vice versa). The P/N OD ratio is a practical and useful criterion but must be used with full understanding of the following problems. It is often poorly reproducible among operators or between tests performed on different occasions or in different laboratories, and, generally, is not directly proportional to antibody concentration over a wide range of serum dilutions (Burke *et al*., 1985a; Kurstak, 1985). This was also demonstrated by P/N OD ratios for IgG being randomly distributed between two and ten instead of clustering near a particular value for each of several groups of specimens representing serially increasing levels of Nt titer (Johnson *et al*., 2000) or by OD values varying widely for specimens with the same HI or PRNT titer (Kittigul *et al*., 1998; Shu *et al*., 2002). Similarly, for IgM, an extensive variation of P/N OD values and the lack of a proportional relationship between the OD value and antibody concentration were reported (Tardei *et al*., 2000). When P/N ratios of IgM for two or more flaviviruses endemic in a given location are all above the cut-off but the values for a dominant agent are widely separated from others (i.e., >10 vs <3), identification of the etiologic agent is not difficult. However, depending on the composition of *Flavivirus* endemic in a given area, overlapping ranges of the OD ratio among the viruses would pose a problem unless all relevant antigens are employed for testing every sample (Martin *et al*., 2002). Furthermore, when the highest ratio of a serum specimen corresponds to one virus (i.e., DEN-3) but with a CSF specimen of the same patient it corresponds to another virus (i.e., JE) (Yoshida *et al*., 1999), diagnosis is further complicated, as for example when cases of CNS syndrome associated with DEN have been reported more frequently. It is well recognized that when OD values of the denominator are very small, even though OD values of the numerator are below the cut-off, OD ratios often assume equivocal, high values. Setting a permissible range of OD values for normal control specimens and invalidating the tests in which this value falls outside the range (Johnson *et al*., 2000) is a good practice.

b. Criteria Based on Dose-Response Curve A dose-response curve drawn on a chart may be linear, polynomial, or sigmoidal, depending on the selection of scales. A composite standard curve on a semilogarithmic chart based on OD values of a group of specimens has been often used to extrapolate antibody concentration in titer or unit by using

a single dilution of specimens (Hofmann *et al.*, 1983; Morita *et al.*, 1982). As a variation, the OD ratio was calibrated to units (Burke *et al.*, 1985a). The problems of the application of the dose-response curve are twofold. First, the range of the dose-response curve in which a proportional relationship is observed occurs in a rather narrow range of serum dilutions (Tardei *et al.*, 2000), with ODs of very low and very high dilutions demonstrating greater deviations from the projected relationship. Second, when specimens are examined individually rather than as a composite, slopes at the best range are found to vary considerably among humans. In fact, assays with a single serum dilution do not generally provide information related to parallelism and neoparallelism. Thus, the presence or absence of parallelism between a standard reference serum and a serially diluted sample curve has been the subject of controversy (Plikaytis *et al.*, 1994). To offset the deviations from the standard curve, a variety of modification or transformation techniques have been introduced for curve fitting, but no completely satisfactory method has been found thus far for flavivirus infections.

B. Comparison of Diagnostic Efficacy among Serologic Tests, Sensitivity, Specificity, and Predictive Values

When the same specimens are tested by two or more techniques, discordant results have been observed on some specimens, for example, between PRNT and IFA (Kayser *et al.*, 1985; Monath *et al.*, 1981; Niedrig *et al.*, 1999), IFA and IgM ELISA (Besselaar *et al.*, 1989), HI and IgM ELISA (Burke *et al.*, 1985a), HI and PRNT (Harabacz *et al.*, 1992), and PRNT and IgG ELISA (Broom *et al.*, 1987). The exact cause(s) of the discrepancy in most such reports is often highly complex, requiring the consideration of many factors. Furthermore, except for TBE and YF viruses (Krag *et al.*, 1965; Table I), international reference serum specimens are unavailable. In comparative studies among PRNT, CF, and HI on convalescent phase specimens obtained more than several months after onset of illness, the differences in antibody detection most likely reflect the differences in the length of persistence of antibodies reactive in each test. However, when the immunoglobulin detected, its temporal dynamic and/or the requirement of paired specimen are different between tests, the validity of the comparison itself needs to be re-examined. For example, sensitivities of IgM capture ELISA and HI on JE specimens were 63% and 38%, respectively, during the height of a JE outbreak, but 26% and 33%, respectively, during nonepidemic periods when fewer cases occurred

(Burke *et al.*, 1985a). Because sensitivity and specificity, in contrast to predictive values, should not be affected by prevalence, the aforementioned data clearly show that HI and IgM ELISA should be compared in terms of diagnostic efficacy or predictive value rather than sensitivity and specificity.

As pointed out earlier in an IgM capture EIA (Kuno *et al.*, 1998), how specimens are selected has a great impact on the sensitivity and specificity of the test. For example, tests on blood specimens obtained at outpatient clinics from cases with mild symptoms may yield lower values than those obtained on admission from hospitalized patients as reflected by the sharp contrast in IgM positivity between less than 30% and more than 75% (Chungue *et al.*, 1992; Innis *et al.*, 1989; Kittigul *et al.*, 1998; Nogueira *et al.*, 1992a; Ruechusatsawat *et al.*, 1994). A longer period between illness onset and hospitalization (4.7 days on average, in a report by Pancharoen and Thisyakorn, 2001) would also favor a higher IgM positivity on admission. Selection of positive and negative references in each test also requires a careful consideration. If, for example, in ELISA a strongly positive specimen was used without dilution as positive reference, a higher proportion of weakly positive specimens may become false-negative. Thus, using a known weakly positive specimen (Innis *et al.*, 1989) is a good practice to reduce this possibility. Also, all specimens must be tested blindly when comparing quality between tests.

VII. Commercial Reagents, Test Kits, and Services

The current proliferation and diversity of commercially available reagents, kits, and services for diagnosis and research of flavivirus infections have been remarkable. Table I shows the products and services available as of April 2003. As the problem of *Flavivirus* infections continues to expand globally, more products are expected to enter the market. Some of the kits, in particular, those for DEN diagnosis, have an additional feature to distinguish primary from secondary DEN infection. The distinction between the two kinds of DEN infections is important for those investigating the mechanisms of DHF/DSS pathogenesis and other related topics. However, the most important concern of most physicians attending patients is not so much the ability to segregate the two kinds of infections as to ascertain whether the patients have dengue. Among other developments in progress, human monoclonal and polyclonal antibodies made in transgenic mice, such as anti-JEV MAb (Ishida *et al.*, 2002), may be used in the future as

standardized positive control antibodies in serodiagnostics, besides their primary utility in therapeutics.

Regarding sensitivity and specificity, a considerable variation in quality among some kits has been reported in many publications; other products have been used without proper evaluation. Regardless, it is still the user's responsibility to evaluate quality before applying a kit for diagnostic practice.

Each country's requirements for registration/licensing and marketing of those products and services vary considerably. For example, in the United States, to use animal sera or derivatives used in imported goods (including mouse MAbs used in kits), a permit from the United States Department of Agriculture is necessary. Thus, it is advisable that users check the regulations of the country in which they reside before acquiring products from abroad.

VIII. Diagnostic Strategies

Although reducing the number of false or ambiguous results is the goal in all diagnostic laboratories, given many factors that complicate serodiagnosis, realistically, a portion of any large number of cases will almost always remain inconclusive. Technological advancement has made diagnostic testing simpler, more sensitive, and/or faster. Nevertheless, we are always reminded of the importance of human factors in the decision-making process, because it is not an instrument but a human that ultimately makes the final interpretation based on the combination of clinical and epidemiologic information, patient history (travel, vaccination record, recent record of blood transfusion, and past exposure to flaviviruses), as well as diagnostic laboratory data. Promoting more frequent consultations and even facilitating specimen sharing among laboratories for independent testing on selected important cases or outbreaks is one way to guard against equivocal serologic conclusions that often derive from overconfidence, bias, or lack of necessary reagents with desirable specificity. In other occasions, diagnosticians concluded prematurely either under pressure or for fear of being judged incompetent if definitive diagnosis was not made quickly.

Application of a standardized panel of reagents of indigenous infectious agents in the diagnosis of illnesses for a given region of the world (Martin *et al.*, 2000) may be adequate for most cases, provided that the agents remain confined to endemic or enzootic areas and that the current knowledge of the geographic distribution of arboviruses is accurate. However, lately, reflecting the age of emerging diseases, increasing numbers

of major outbreaks of new or foreign viral diseases in unexpected locations have resulted in initially erroneous serodiagnoses involving or implicating a *Flavivirus* (Enserink, 1999; Holloway, 2000). The expansion of JE-affected regions now includes northern Australia, and invasion of an African virus, Usutu, to Europe was recently reported (Weissenböck *et al.*, 2002). As for the second subject, the reports of isolation of RSSE virus from a large number of rodents, humans, and ticks in southern China bordering Myanmar (Burma) (Hou *et al.*, 1992; Huang *et al.*, 1992; Zi *et al.*, 1990) clearly indicate that the geographic distribution of this virus recognized in the past was not entirely accurate. Thus, it is clear that constant monitoring of the distribution of many medically important arboviruses is imperative. Accordingly, in addition to the above standardized strategy for a rapid diagnosis of viral syndrome cases of unknown origin, establishment of an alternative diagnostic strategy using carefully selected reagents of exogenous viruses representing a broader range of arboviruses and other animal virus groups has become necessary. Deploying those two strategies simultaneously, whenever deemed necessary, rather than sequentially is required at least in major laboratories with sufficient resources.

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ADVANCES IN VIRUS RESEARCH, VOL 61

MOLECULAR AMPLIFICATION ASSAYS FOR THE DETECTION OF FLAVIVIRUSES

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Over the past 10 years, a number of molecular amplification assays have been developed for the detection of flaviviruses. Most of these assays utilize the reverse transcriptase-polymerase chain reaction (RT-PCR) as the amplification format with detection by either agarose gel electrophoresis and ethidium bromide staining or hybridization with molecular probes. Recently, a modification of the standard RT-PCR using fluorescent-labeled oligonucleotide probes for detection (TaqMan) has been described. As a result, several assays for detecting flaviviruses have been developed using this approach. In addition, another amplification format, nucleic acid sequence based amplification (NASBA), has been developed and utilized for the detection of several flaviviruses. The various assay formats will be described and their utility discussed.

I. Introduction

The development of the polymerase chain reaction (PCR) in conjunction with the discovery of thermostable DNA polymerases in the late 1980s revolutionized the field of viral diagnostics (Mullis *et al.*, 1987;

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Saiki *et al.*, 1988). Before 1985, virus detection assays consisted primarily of virus isolation in tissue culture or mice followed by immunofluorescence assay (IFA), direct staining (IFA or immunoperoxidase) of cells or tissues, electron microscopy, and antigen capture enzyme-linked immunosorbent (ELISA) assays. Virus isolation followed by IFA generally requires more than 1 week to complete and depends on virus survival in the clinical specimen. Electron microscopy is technically difficult, requiring specialized expertise, reagents, and expensive equipment, and is also not amenable to testing large numbers of samples. Antigen capture ELISA assays are capable of testing large numbers of samples; however, these assays are generally not as sensitive as virus isolation in tissue culture. The nucleic acid detection assays in use before PCR consisted mainly of direct detection assays, primarily filter-based hybridization assays (i.e., dot blot, slot blot) using virus-specific probes. These assays frequently used radioisotopes (^{32}P-labeled probes) and were technically difficult and therefore of limited utility to the viral diagnostic laboratory.

In the 1990s, a number of reverse transcriptase-PCR (RT-PCR)–based assays began to be described in the literature for flaviviruses. These offered sensitivity comparable to virus isolation in tissue culture yet with a much more rapid turn around time. In addition, the amplified DNA could be subjected to nucleic acid sequence analysis, which removed all ambiguity in the identification of the virus. Furthermore, the nucleic acid sequence data could also provide strain information about the virus. This chapter will attempt to summarize assays that have specifically been designed for the detection of flaviviruses. It is important to note at the outset that virus isolation continues to be the "gold standard" for virus detection assays. In my opinion, virus isolation should always be used in conjunction with all of the newer molecular amplification assays so that virus strains can be isolated, further characterized, and cataloged in reference collections.

II. Overview of Molecular Amplification Technologies

All molecular amplification assays involve three basic steps: 1) nucleic acid extraction/purification from samples; 2) amplification of the nucleic acid; and 3) detection/characterization of the amplified product (Fig. 1). In the case of flaviviruses, the first step is extraction and purification of RNA from a variety of specimen types, which could include human serum, human cerebrospinal fluid (CSF), homogenized mosquito pools, homogenized ticks, or tissues. The various RNA extraction methods currently in use will not be discussed in detail here;

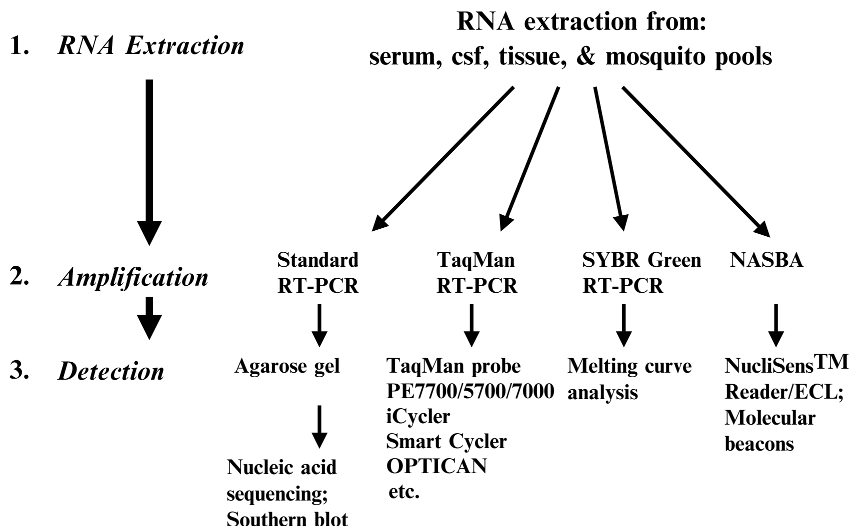


FIG 1. Overview of the three steps used in most molecular amplification assays (RNA extraction, amplification, and detection) and the various amplification and detection technologies. Listed under the TaqMan detection format are the various instruments currently available.

however, it is important to note that there has been considerable improvement in the extraction and purification of RNA over the past 10 years. Traditional methods of extraction involving organic liquid phase separation (i.e., phenol, chloroform, etc.) are being gradually replaced by commercial kits that use silica (either bound to beads or to columns) to bind nucleic acids with subsequent washing. For a comparison of the various RNA extraction methods currently in use, see Verhofstede *et al.* (1996). The combined effect of the use of kits has been a more reproducible and rapid RNA extraction method in which larger numbers of samples can be processed with greater ease. In addition, the silica-based commercial kit extraction protocols can also be automated by using liquid handling robotics systems, such that over 500 samples can be readily processed in one work day (Shi *et al.*, 2001).

A. Standard Format RT-PCR–Based Assays

The most common amplification assays for the detection of flaviviruses, and among the first to be developed, use a standard RT-PCR amplification format. The standard RT-PCR reaction involves two steps: reverse transcription of viral (plus sense) genomic RNA into

single-stranded DNA (cDNA), followed by conversion to double-stranded (ds) DNA and amplification of the ds DNA by Taq polymerase. These two steps can either be performed in two separate reactions (two-step RT-PCR), using two sets of reagents, or in a single reaction vessel containing both reverse transcriptase and Taq polymerase enzymes using common reagents (1-step RT-PCR). Most assays currently use the latter approach because of rapidity and ease of reaction set-up. Single-step RT-PCR kits are also commercially available from a number of vendors. Reverse transcription can be achieved through the use of any one of the several commercially available retroviral reverse transcriptase enzymes (MuMLV, AMV, rav, etc.), or in the case of Tth enzyme, the enzyme can be used both for RT and the subsequent PCR. The RT reaction can use either a virus-specific oligonucleotide to prime DNA transcription, or alternatively, random hexamers can be used to initiate transcription. In some cases, the use of random hexamers achieved a greater overall sensitivity in the detection of viral targets (Howe *et al*., 1992). There are also a number of commercially available thermostable polymerases that can be used for the PCR amplification. These enzymes differ in properties such as thermal stability, fidelity (i.e., error rate), exonuclease activity (5′ to 3′ and 3′ to 5′) and others. Several reviews describe the various Taq polymerase enzymes currently available along with their advantages and disadvantages (Abu Al-Soud *et al*., 1998; Cline *et al*., 1996).

Following the RT-PCR amplification, some type of detection method must be used to determine if amplification occurred and to characterize the amplification product. The most often used method involves determining the size of the amplified DNA product by electrophoresis on an agarose gel with visualization by staining of the DNA using the dye ethidium bromide. In this approach, obtaining a DNA fragment of the predicted size (molecular weight) is considered by some to be diagnostic. However, it is important to note that in some instances nonspecific amplification can generate DNA products with similar or identical mobility on agarose gels to the predicted fragment. This in turn would lead to false-positive interpretation of results. Another more recent method of detecting amplified DNA involves the use of the DNA binding dye SYBR Green, in which fluorescence of the dye increases when bound to DNA. The dye is included in the RT-PCR amplification reaction, and the accumulation of amplified DNA is detected in real time by measuring an increase in fluorescence. This approach requires the use of a thermocycler that is able to measure fluorescence during temperature cycling; a number of these instruments are currently available (see later). At the conclusion of the amplification, the instrument

calculates the melting temperature (Tm) of the amplified DNA(s), so that the predicted DNA product can readily be distinguished from other nonspecific amplified DNA-products (i.e., primer dimers). Using this approach, assays have been developed that detect and identify enteroviruses and herpesviruses in CSF specimens based on the Tm of the amplified DNA (Read *et al.*, 2001). This approach has also been successfully implemented in our laboratory using consensus primers for flavivirus detection and holds great promise for the development of new assays.

Sequence-specific approaches for detecting and confirming the identity of the amplified DNA include hybridization with virus-specific probes (i.e., Southern blot, dot-blot, or microtiter plate hybridization), PCR amplification with additional primers internal to the original primers (nested or semi-nested PCR), restriction endonuclease digestion of the DNA product, or nucleic acid sequence analysis. In general, the probe hybridization methods are technically difficult and time-consuming. However, by using a 96-well format for the hybridization assay, large numbers of samples can be processed. Nested or semi-nested PCR offers the advantage of increasing sensitivity along with confirmatory identification, yet extreme diligence in laboratory technique must be maintained to avoid amplicon contamination and the generation of false-positive results. Digestion with restriction enzymes has been used in several cases; however, a single base substitution could lead to false-negative results. Nucleic acid sequence analysis clearly offers the most unambiguous characterization of the amplified DNA, but the procedure can be time-consuming and difficult to process large numbers of samples. However, new instrumentation, in particular capillary nucleic acid sequencers that allow for the sequence analysis of large numbers of samples, has moved nucleic acid sequencing into the battery of tests that can be readily performed in the clinical diagnostic laboratory.

With respect to primer design, the RT-PCR assay can use either virus-specific primers or consensus primers that are designed to amplify genetically related viruses. In either case, primer design should include the analysis (alignment) of as many sequences as available so that the assay will achieve the desired result: either high specificity or broad reactivity. For example, consensus primers able to amplify all four dengue serotypes have been developed by a number of investigators by designing primers in regions that are conserved among the four dengue serotypes (Henchal *et al.*, 1991; Lanciotti *et al.*, 1992; Wang *et al.*, 2000). In some cases, consensus primers are synthesized that possess more than one base at a given position (i.e., A or T) to

accommodate sequence variation (degenerate primers). This is the case for consensus primers that have been designed to amplify all flaviviruses (Chang *et al.*, 1994; Scaramozzino *et al.*, 2001). When consensus primers are used, detection methods in addition to agarose gel electrophoresis (see previously) must be used to specifically identify the resulting DNA, since by the design of the assay all related viruses would all be amplified.

B. Real Time 5′ Exonuclease Fluorogenic Assays (TaqMan)

Recent advances in RT-PCR amplification methods, which hold a great deal of promise for highly specific and sensitive virus detection assays, combine RT-PCR amplification with fluorescent-labeled virus-specific probes able to detect amplified DNA during the amplification reaction. These assays offer numerous advantages over standard RT-PCR; namely, increased sensitivity and specificity, quantitation, high throughout, and rapid turnaround of results. All of these advantages are due to the nature of the amplification reaction and the availability of instruments able to perform both thermocycling and fluorescence detection (for a detailed review of TaqMan chemistry, see Bustin, 2000). The overall scheme of the TaqMan amplification strategy is shown in Fig. 2. The RT and PCR amplification reaction is identical to that described above in the standard format RT-PCR. However, included in the reaction along with the two primers is a virus-specific oligonucleotide probe, dual labeled with a fluorescent reporter dye (R in Fig. 2) and a quencher molecule (Q in Fig. 2). When the oligonucleotide probe is intact, either free in solution or bound to target, the reporter and quencher are in close proximity and the emission fluorescence from the reporter dye is quenched. The Taq polymerase enzymes utilized in these TaqMan assays (either Taq or Tth) possess a 5′ to 3′ exonuclease activity, and as a result, during replication of one of the DNA strands the enzyme will encounter the bound probe and cleave it, resulting in the release of the reporter dye into solution. The release of the reporter dye and its physical separation from the quencher molecule results in an increase in fluorescence measurable by the instrument. The cycle number at which fluorescence exceeds background fluorescence is referred to as the threshold cycle (Ct) (Fig. 3A). The Ct value is the key parameter in evaluating the qualitative result of the assay, and in addition, it is directly proportional to the original amount of RNA target present in the reaction. Therefore, by including quantitated RNA standards of the same viral RNA target tested for in the experiment, a standard curve can be

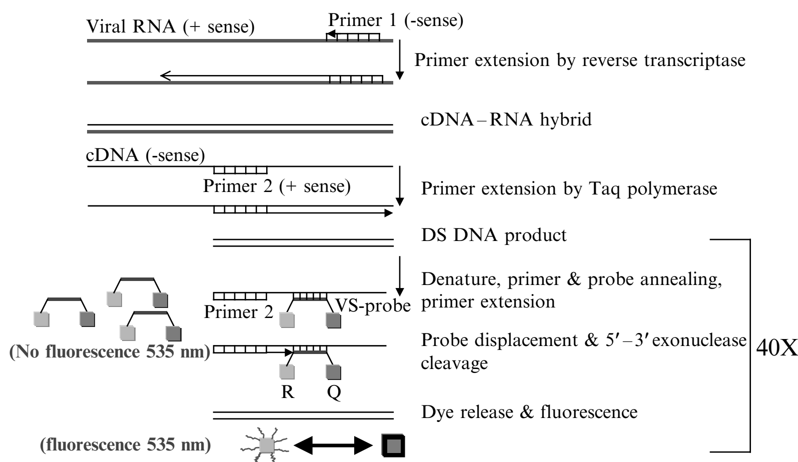
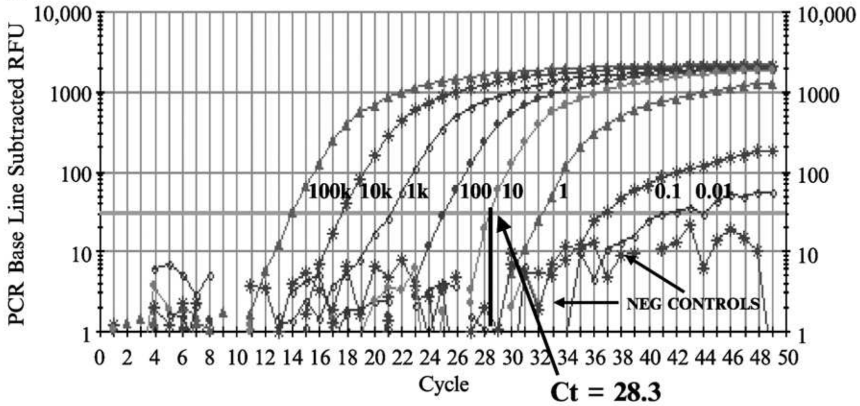


FIG 2. Schematic overview of TaqMan RT-PCR amplification. A cDNA copy (black line) of the viral positive sense RNA (red line) is synthesized by reverse transcriptase using primer 1 to initiate the reaction. The second DNA strand (black line) is synthesized by Taq polymerase using primer 2. The TaqMan oligonucleotide probe is complementary to the negative sense strand of the amplified DNA and is dual labeled with a reporter dye (FAM-green) and a quencher molecule (magenta). During extension by Taq polymerase, the 5′ to 3′ exonuclease activity of Taq polymerase hydrolyzes the bound probe resulting in the release (and physical separation) of the two dyes. The separation of the dyes can be detected by an increase in fluorescence at the emission wavelength of the reporter dye—in the case of FAM at 535 nm. (See Color Insert.)

generated by plotting the threshold cycle (Ct) vs the RNA target quantity. The standard curve can then be used to estimate the starting quantity of viral RNA in the test specimens. Figure 3 shows the amplification plot (A) and the resulting standard curve (B) obtained by performing the assay on a dilution series of WN virus that was previously quantitated by plaque titration.

The increased specificity of the TaqMan assay compared to standard RT-PCR is due to the use of the virus-specific internal probe during the amplification. The hybridization of this probe to the target sequence and subsequent hydrolysis is detectable by the increase in fluorescence. This sequence-specific detection obviates any post-amplification characterization of the amplified DNA. As a result, amplified DNA is not manipulated in the laboratory as occurs with standard RT-PCR, thus greatly reducing the likelihood of amplicon contamination.



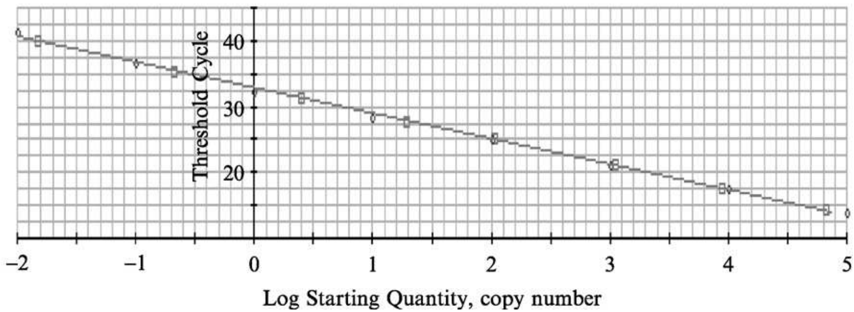


FIG 3. Typical data obtained from a TaqMan RT-PCR assay performed on the BioRad iCycler. (A) Amplification plot of a ten-fold dilution series of WN viral RNA. The y-axis is the relative increase in the reporter dye fluorescence (RFU = relative fluorescence units); the x-axis is the cycle number of the polymerase chain reaction. The key parameter used in determining both the qualitative and quantitative result of the assay is the Ct (threshold cycle). The Ct is defined as the cycle number at which fluorescence increases above the threshold fluorescence (horizontal red line); in this example Ct = 28.3. Displayed adjacent to each amplification curve is the starting quantity of virus in plaque forming units (100 k = 100,000 pfu etc.). Negative controls are shown and are all below the threshold fluorescence line. (B) Standard curve of the TaqMan assay generated by plotting the Ct (y-axis) against the log of the starting quantity of virus in pfu. The standards (blue) and the unknown samples (red) are displayed in the graph. (See Color Insert.)

Real-time fluorogenic assays also offer the advantage of the ability to detect multiple targets at the same time in the same amplification reaction (multiplexing). This can be accomplished by using multiple oligonucleotide probes (up to four in some instruments) each labeled with

fluorescent reporter dyes with discrete emission spectra. For example, internal positive RNA controls can be co-amplified along with the target RNA to monitor the efficiency of the RT-PCR reaction (Fig. 4). Alternatively, testing for more than one viral target in a single reaction can also be accomplished through multiplexing. In this laboratory all four dengue serotypes can be amplified and detected in a single reaction through the use of dengue type–specific probes labeled with the fluorescent dyes FAM, HEX, Texas Red, and CY5.

A number of instruments are currently available that are able to perform thermocycling in combination with fluorescence detection. In addition to TaqMan assays, these instruments can also be used to perform other assay formats using fluorescent dyes, such as molecular beacons and SYBR Green detection in real time (see previously). The Applied Biosystems 7700 and its progeny the 7000 can excite and detect fluorescent dyes up to the mid/upper 500 nm range, and multiplexing

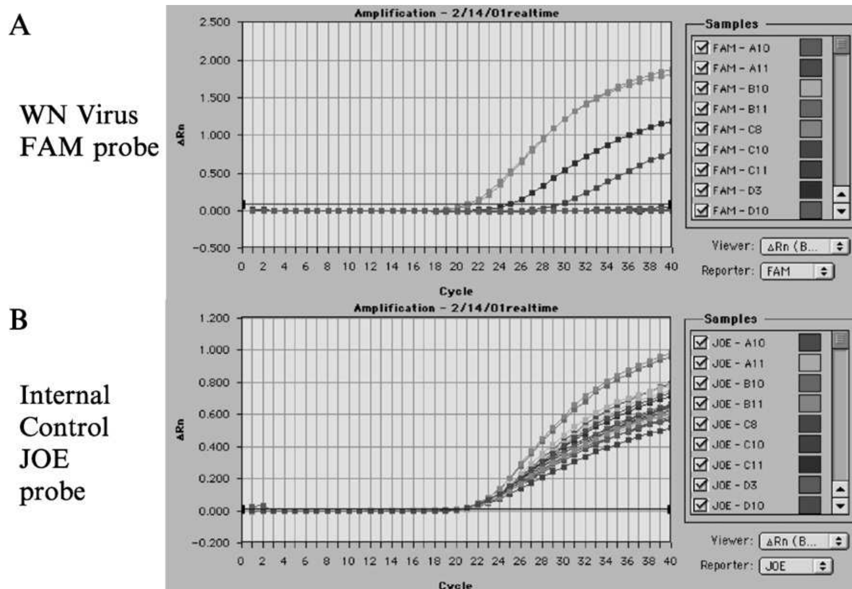


FIG 4. Typical data obtained from a TaqMan RT-PCR assay performed on the ABI 7700 testing 96 mosquito pool specimens for the presence of WN virus. Panel A displays the amplification plot using the WN specific FAM labeled probe; note that four of the 96 specimens are positive for WN viral RNA. Panel B as displays the amplification plot using the internal RNA control JOE labeled probe; note that all 96 specimens are positive, indicating that reagent and thermocyling conditions were optimal for amplification.

up to two dyes in these instruments has been reported. The DNA Engine Opticon (MJ Research) is a single dye instrument able to detect FAM or SYBR Green. The iCycler (BioRad), the Mx4000 (Stratagene), the RotorGene (Corbett Research), and the SmartCycler (Cepheid) are all able to excite and detect dyes up to around 800 nm. As a result, multiplexing can use dyes ranging from FAM (emission 525 nm) to CY5 (emission 670 nm), and assays using four dyes in a single reaction have been demonstrated. In our laboratory, direct comparison of the 7700, 7000, iCycler, and the OPTICAN yielded very similar results.

C. Nucleic Acid Sequence–Based Amplification (NASBA)

Another amplification technology that has successfully been used for the detection and identification of flaviviruses is NASBA (Fig. 5). This approach shares some similarities with RT-PCR at the initial

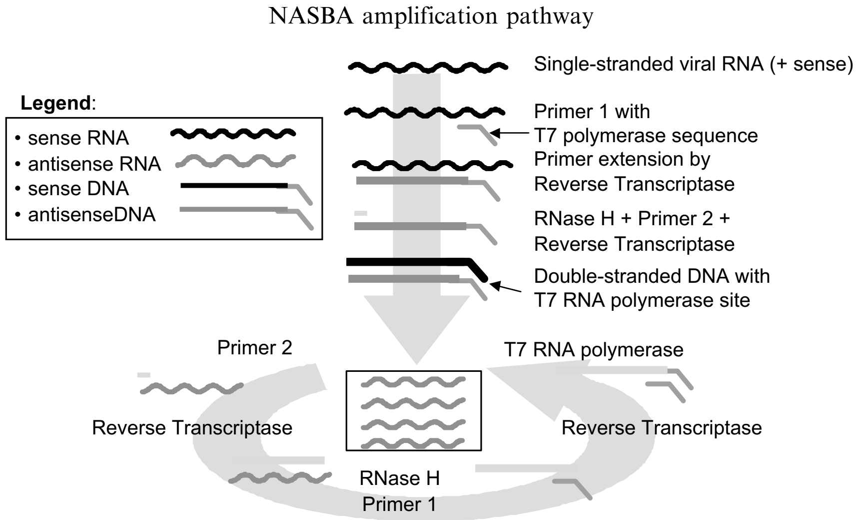
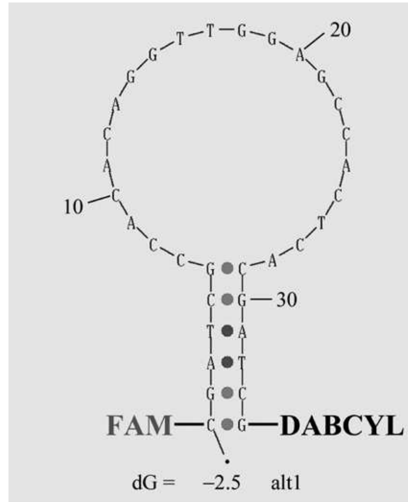


FIG 5. Schematic overview of NASBA amplification. A cDNA copy (purple line) of the viral positive sense RNA (black curved line) is synthesized by reverse transcriptase using primer 1 to initiate the reaction. Primer 1 has a T7 RNA polymerase recognition sequence at the 5′ end, which is incorporated into the nascent cDNA. The second DNA strand (black line) is synthesized by reverse transcriptase. The resulting double-stranded DNA has an intact T7 RNA polymerase promoter sequence, which is used by T7 RNA polymerase to generate RNA transcripts (purple curved lines). These single-stranded RNA products can serve as templates to generate more double-stranded DNA resulting in amplification of the RNA. (See Color Insert.)

stages; however, there are several significant differences: the reaction is isothermic (41°C); the enzymes used are reverse transcriptase, RNase H, and T7 RNA polymerase; and the final amplification product is single-stranded RNA. The initial stage of NASBA is an RT reaction in which single-stranded viral RNA target is copied into a double-stranded DNA molecule using RT and two virus-specific primers (Fig. 5). The reverse sense primer possesses a 5′ non-target sequence consisting of a T7 polymerase binding motif. In the electrochemiluminescence (ECL) detection format developed by Organon Teknika (see below), the forward primer also has a 5′ non-target region consisting of a sequence complementary to the kit's ECL detector probe. As a result, at the conclusion of second strand synthesis the resulting double-stranded DNA possesses an intact T7 RNA polymerase binding sequence at the 5′ end, and an ECL detector probe binding sequence at the 3′ end. The ECL detector probe binding sequence allows for the use of a ruthenium-labeled generic detector probe for all ECL based NASBA assays regardless of the viral target of the assay. This double-stranded DNA can now serve as a template for RNA transcription by T7 RNA polymerase. The resulting single-stranded RNA can then in turn serve as new templates for cDNA synthesis via the RT reaction so that the amplification cycle can proceed. The net effect is the rapid and large accumulation of single-stranded RNA products of opposite polarity to that of the original target. As with RT-PCR, detection of amplification can be achieved through agarose gel electrophoresis with ethidium bromide staining; however, this approach suffers from the same lack of specificity as that described for RT-PCR. A more sequence-specific and sensitive approach that is commonly used involves the use of a virus-specific capture probe bound to magnetic beads in solution. The captured RNA is then detected by using a ruthenium labeled detector probe that has a sequence complementary to the 3′ end of the amplified RNA-the ECL detector sequence. The chemiluminescence is then measured in a semi-automated instrument (NucliSens Reader, BioMerieux). This approach has been used successfully for the detection of a number of RNA viruses including WN, SLE, and dengue viruses (Lanciotti *et al.*, 2001; Wu *et al.*, 2001). Alternatively the amplified RNA can be detected in real time with the use of a virus-specific molecular beacon probe using a fluorescent dye as the detector molecule. The molecular beacon possesses an internal virus-specific sequence flanked by a self-complementary region so that a stem-loop structure is formed in the absence of target (Fig. 6). The 5′ and 3′ ends are labeled with a reporter dye and quencher molecule so that in the stem loop configuration no fluorescence is detected. As the



WN Molecular Beacon Probe

FIG 6. Primary nucleotide sequence and predicted secondary structure of the WN virus molecular beacon probe. Folding algorithm by D. Stewart and M. Zucker; © 2001 Washington University. (See Color Insert.)

amplified RNA accumulates in the reaction, the beacon probe binds to the target, resulting in a measurable increase in fluorescence detected by the instrument.

D. Sensitivity and Specificity of Molecular Amplification Assays

The key criteria for evaluating any new virus detection assay is that of sensitivity and specificity. In general terms, sensitivity is a measure of the limit of detection of the assay—how few viral targets are necessary to produce a positive result. In some cases it is difficult to directly compare assays described in the literature due to the variety of units used to define virus quantities. For example, RNA copy number, plaque-forming units (pfu), weight of RNA (pg, fg, etc.), and others have all been used. When describing viral quantities in pfu, it is also important to consider that particle to infectivity ratios vary among flaviviruses; all would be expected to be in the range of 100 to 1 or greater. For example, it has been calculated that approximately 500 viral particles (RNA copies) are necessary to produce 1 pfu of WN (Shi *et al.*, 2001). Therefore, when an assay demonstrates the ability to detect 0.1 pfu of WN virus, this corresponds to approximately 50

RNA genome copies, which would also be equivalent to approximately 0.3 fg of RNA.

It is also important to consider that in the standard RT-PCR format, in which visual observation of the stained gel is used in the final analysis for qualitative interpretation, subjectivity is introduced in defining what constitutes a positive result. Nationwide proficiency testing conducted by the Centers for Disease Control for WN testing using standard RT-PCR revealed a significant lab-to-lab variation in the subjective interpretation of agarose gel visual results. In contrast, the newer more automated amplification technologies, NASBA and TaqMan, rely on instrumentation to detect the presence of amplified nucleic acid. As a result, lab-to-lab differences in this case would be expected to be minimal. In the same proficiency testing project, lab-to-lab variation using TaqMan or NASBA were in fact minimal, and in the case of TaqMan the calculated viral quantities varied at most by 5% between laboratories.

Another component in evaluating the sensitivity of a published viral diagnostic assay is the ability of the test to detect the viral target in specimens similar or identical to what would be encountered in the diagnostic laboratory. In most cases described in the literature, the sensitivity of an assay is evaluated by using known amounts of virus seed produced in the laboratory. This may not be an accurate measure of the ability of the assay to detect viral RNA in "real world" specimens in which the target viral RNA is present in a background of large amounts of cellular nucleic acid. A better approach used by some is the testing of "spiked samples." In this case known amounts of virus are added to negative clinical samples (i.e., serum or CSF). The best scenario for evaluating the sensitivity of a new assay is to test clinical samples that have been tested and/or quantitated by other "gold standard" tests such as virus isolation, or samples in which infection can be verified by serologic tests. With many flaviviruses, however, large numbers of clinical samples are rarely available and the use of spiked samples is the best achievable option.

The specificity of a molecular amplification test is a measure of the ability of the assay to specifically amplify and detect a particular viral RNA while producing negative results with other nucleic acid targets. Of particular importance (if this is the intent) is the ability of the assay to detect all strain variants of the viral target. Most investigators use nucleic acid database searches in their assay (primer) design to achieve this aim. The nucleic acid sequences of multiple strains of a particular virus are aligned and primers are designed in regions where complete nucleic acid sequence conservation is observed. In addition,

designed primers should be analyzed for sequence similarity to related viruses and/or host nucleic acid sequences through database searches (i.e., GenBank BLAST searches). However, even with such analysis it is important to evaluate the primers by using them in the molecular assay with strain variants and related viruses to ensure that all variants are detected and that related viruses produce negative results. This evaluation should also include the testing of either "real world" specimens or "spiked" specimens because most specimens encountered in the laboratory will contain large amounts of non-target RNA.

E. Testing Algorithms, Data Analysis, and Interpretation

The establishment of sound testing algorithms for molecular amplification assays is necessary to ensure that results have a high degree of confidence. The most desirable testing algorithm is one in which the molecular amplification assays are complemented by other virus detection assays, in particular virus isolation in tissue culture since this is still considered by most as the "gold standard" assay. The benefit of this approach is that virus isolates would be generated, cataloged, and thus available for further characterization. The other strength of this approach is that new viruses and/or strain variants would be isolated, whereas these may not be detected in virus specific molecular assays. Other non-amplification–based "direct tests" such as antigen capture ELISA or direct IFA are very useful in confirming molecular amplification results, although such tests are typically not as sensitive as either isolation or amplification-based tests.

In addition, the molecular assay itself must incorporate the appropriate controls to ensure against false-positive or false-negative results. At a minimum, clinical samples should be tested in duplicate and positive specimens confirmed in a follow-up test with a second set of primers derived from a different region of the genome. Positive controls included in each assay should contain various levels of target RNA (high, medium, low, etc.) to evaluate the sensitivity of each test in comparison with previous tests. The use of an internal positive control can also be very useful when testing specimens that may contain inhibitors of RT-PCR (i.e., blood, mosquito lysates, etc.). In the WN TaqMan assay used at the CDC, an artificial RNA molecule is transcribed from a plasmid vector and added to each sample. The internal control RNA is co-amplified along with the target RNA, and detected with a probe labeled with a different fluorescent dye (JOE) than that used by the target RNA, so that amplification of both targets can be measured simultaneously. Figure 4 displays an example of an assay

in which 96 mosquito specimens were tested for WN virus RNA (A) along with the internal control RNA (B). As can be observed, only a few samples (4) are positive for WN; however, all specimens are positive for the internal control with approximately the same Ct, indicating that conditions existed for the efficient amplification of each tested specimen.

As with all amplification based technologies, it is essential that proper laboratory procedures be followed which prevent cross contamination of nascent samples with amplified DNA (amplicon) from previous tests, resulting in the generation of false-positive results. Details of the appropriate laboratory protocols will not be discussed here, but in general there should be complete physical separation of pre- and post-amplification laboratory space and equipment. Of particular importance is the use of several no template controls (NTC) spaced randomly throughout the assay to monitor for amplicon contamination. If any of the NTCs produce positive results, the entire run should be considered invalid.

III. RT-PCR–Based Assays for Flaviviruses

A. *Flavivirus Consensus Assays*

There are a number of reports in the literature describing the detection of multiple flaviviruses using a standard format RT-PCR by using consensus primers (Table I). In these assays, primers are designed in regions of the flavivirus genome that possess a high degree of sequence conservation. This approach not only offers a convenient method of screening large numbers of samples for flaviviruses, but in addition, the ability exists to potentially detect new or variant flaviviruses. The rationale is that by designing primers able to amplify all currently known flaviviruses, the likelihood of identifying new or related viruses would be greatly increased. The majority of these consensus protocols utilize primers in either the terminal portion of the NS5 gene or the 3′ non-coding region, due to the existence of several highly conserved regions in this portion of the viral genome (Chang *et al.*, 1994; Figueiredo *et al.*, 1998; Fulop *et al.*, 1993; Kuno, 1998; Pierre *et al.*, 1994; Scaramozzino, *et al.*, 2001; Tanaka, 1993). Interestingly, three of the five publications in which NS5 primers were used all utilized a downstream primer that is either identical to or within five nucleotides of one another (Chang *et al.*, 1994; Kuno, 1998; Scaramozzino *et al.*, 2001). The remaining protocols use primers directed to either the

TABLE I

RT-PCR Assays Using Primer Pairs for Detecting Multiple Flaviviruses

Reference	Primer location[a]	Amplification format	Detection format	Flaviviruses detected[b]	Detection limit[c]
Scaramozzino *et al.*, 2001	NS5	2-step RT-PCR + semi-nested	agarose & SYBR Green	DEN-1 DEN-2 DEN-3 DEN-4 JE WN YF USU LAN ZIK WSL	200 TCID/mL
Figueiredo *et al.*, 1998	NS5/3′NC[d]	1-step RT-PCR	agarose	DEN-1 DEN-2 DEN-4 CAC IGU ILH ROC SLE YF[e]	ND[f]
Kuno, 1998	NS5	1-step RT-PCR	agarose	All 66	ND
Meiyu *et al.*, 1997	NS1	2-step RT-PCR + semi-nested	agarose	DEN-1 DEN-2 DEN-3 DEN-4 JE LAN POW	ND
Puri *et al.*, 1994	ENV	2-step RT-PCR	agarose	DEN-2 WN SLE KUN[g]	1 pg RNA
Pierre *et al.*, 1994	NS5/3′NC	2-step RT-PCR	dot-blot hybridization	DEN-1 DEN-2 DEN-3 DEN-4 JE WN YF ZIK	5 pfu DEN-2
Chang *et al.*, 1994	NS5	1-step RT-PCR	microtiter hybridization	DEN-1 DEN-2 DEN-3 DEN-4 JE YF SLE	10 pfu YF

Chow *et al*., 1993	NS3	2-step RT-PCR	agarose	DEN-1 DEN-2 DEN-3 DEN-4 JE YF KUN	0.1 pg RNA DEN-3
Fulop *et al*., 1993	NS5	2-step RT-PCR	agarose	DEN-2 JE YF ILH KUN WSL TBE MVE POW BAN MOD RIO	ND
Tanaka *et al*., 1993	NS5/3′NC	1-step RT-PCR	agarose	DEN-1 DEN-2 DEN-3 DEN-4 JE YF SLE WN MVB	ND

[a] Gene abbreviations: NS5 = non-structural 5; 3′NC = 3′ non-coding region; NS1 = non-structural 1; ENV = envelope; NS3 = non-structural 3.

[b] Virus abbreviations: DEN = dengue; JE = Japanese encephalitis; WN = West Nile; YF = yellow fever; USU = Usutu; LAN = Langat; ZIK = Zika; WSL = Wesselsbron; CAC = Cacipacore; IGU = Iguape; ILH = Ilheus; ROC = Rocio; SLE = St Louis encephalitis; KUN = Kunjin; TBE = tick-borne encephalitis; MVE = Murray valley encephalitis; POW = Powassan; BAN = Banz; MOD = Modoc; RIO = Rio Bravo.

[c] TCID = tissue culture infectious doses; pfu = plaque forming units.

[d] Same primers as Tanaka *et al*., 1993.

[e] Did not amplify Bussuquara virus.

[f] ND = not determined.

[g] Did not amplify DEN-1, DEN-3, DEN-4, JE, and YF.

envelope gene, NS1, or NS3 (Chow *et al.*, 1993; Meiyu *et al.*, 1997; Puri *et al.*, 1994). In several instances, primers are synthesized with more than one nucleotide at a given position to accommodate the various nucleotide substitutions observed at these sites (degenerate primers). In general, we have observed a reduction in sensitivity when using consensus/degenerate primers when compared to virus specific non-degenerate primers. In direct comparisons we have observed a 10- to 100-fold loss of sensitivity with both WN and EEE virus assays.

The majority of these flavivirus consensus protocols use agarose gel electrophoresis as the detection platform for analysis of amplified DNA. Confirmatory, sequence-specific identification is incorporated in only two of these assays (Chang *et al.*, 1994; Pierre *et al.*, 1994). These use either dot-blot hybridization or microtiter plate-based hybridization with virus-specific probes. In all cases, nucleic acid sequencing is an option that could be used to identify the amplified DNA product. While all of these publications assert that the assay detects "flaviviruses," only one actually tested the consensus primers against all 66 known flaviviruses (Kuno, 1998). The remaining assays were evaluated by testing a panel of flaviviruses consisting of viruses from most of the serocomplexes; however, in a few cases the assays were evaluated by testing very limited numbers of flaviviruses (Table I).

The comparative sensitivity of these flavivirus consensus assays is difficult to evaluate because only half of the assays displayed in Table I performed any testing on known quantities of virus. Of those that tested quantitated virus, various units to define viral quantity were used, making direct comparisons difficult. In addition, in most cases, sensitivity data with only one viral target was determined. As a result, it is difficult to predict the detection limit of the assay with the other flaviviruses. Because of the sequence divergence of these viruses, it would be expected that sensitivity would vary considerably among the various flaviviruses.

Recently most of the primer pairs displayed in Table I were evaluated for their ability to detect a diverse panel of flaviviruses, all at a fixed concentration of 10^5 50% tissue culture infectious doses per milliliter ($TCID_{50}$/mL) (Scaramozzino *et al.*, 2001). At this concentration, several flaviviruses were not amplified by primers that were previously shown to amplify these viruses. This discrepancy could be the result of testing $>10^5$ $TCID_{50}$/mL in the original report. In this comparison the primers designed by Kuno (1998) were found to be the only pair that successfully amplified all 66 of the flaviviruses. However, these primers were not able to detect any of the flaviviruses when the starting concentration was lowered to 10^4 TCID/mL. As a result,

Scaramozzino (2001) modified one of these primers and introduced a third primer to be used in a semi-nested PCR. Using this adapted protocol, it was possible to detect a broad panel of flaviviruses at a concentration of 200 $TCID_{50}$/mL; however, not all 66 flaviviruses were tested. Therefore the ability of this modified assay to amplify all flaviviruses is unknown.

Another approach described by Scaramozzino (2001) is the use of flavivirus consensus primers in a real-time RT-PCR assay that incorporates the DNA binding dye SYBR Green. The accumulation of amplified DNA during PCR is detected by measuring the increase in fluorescence of the dye as it binds to double stranded DNA with an instrument which combines fluorescence detection with thermocycling. In addition, the specificity of the DNA product is evaluated through melting curve analysis (see previous discussion). The use of SYBR Green in conjunction with instruments capable of measuring fluorescence has demonstrated equal or greater sensitivity than visualizing DNA by ethidium bromide staining on agarose gels (Scaramozzino *et al.*, 2001). The use of this approach has demonstrated a sensitivity similar to that of virus specific TaqMan assays able to detect less that 1 pfu of virus (Lanciotti, unpublished results). In addition, DNA can be recovered and subjected to nucleic acid sequencing for final identification and further characterization of the flavivirus in the original sample.

B. Virus-Specific Tests

1. Yellow Fever Virus

Rapid laboratory diagnosis of YF virus infection is essential to allow for timely implementation of emergency vaccination campaigns. Virus detection assays are often used in the laboratory to confirm YF virus infection because serologic assays may be difficult to interpret in the case of secondary flavivirus infections. YF virus has been detected in acute serum specimens by either antigen capture ELISA or virus isolation (Monath, 1987). Alternatively RT-PCR assays for YF virus have been used for the detection of RNA in acute serum specimens or liver tissue from fatal cases (Deubel *et al.*, 1997). Three RT-PCR assays specific for YF virus detection have been described (Brown *et al.*, 1994; Deubel *et al.*, 1997; Eldadah *et al.*, 1991). All of these systems use virus-specific primers derived from conserved regions of the envelope glycoprotein and the assays were designed to amplify all known YF virus strains. These systems also use a standard two-step RT-PCR amplification format with detection by agarose gel

electrophoresis and ethidium bromide staining. Eldadah (1991) describes detection of the YF 17D strain only, and does not provide any sensitivity data. Brown (1994) reports amplification of YF RNA from 32 temporally and geographically (new and old world) distinct YF strains. In addition, as little as 30 pfu of YF virus were detected in spiked human serum specimens and YF virus was also successfully detected from blood samples obtained from experimentally infected monkeys.

2. Tick-Borne Encephalitis Serocomplex Viruses

The viruses in this serologic complex that have been associated with significant human disease are primarily TBE-Central European (TBE CE), TBE-Far Eastern (TBE FE; i.e., Russian Spring Summer Encephalitis), and Powassan. Other TBE complex viruses known to cause human and/or veterinary disease are Langat, Kyasanur Forest Disease, Louping ill, Omsk hemorrhagic fever, and Negishi viruses. In general, viremia with TBE complex viruses is of short duration, and as a result laboratory diagnosis of human infections is most often achieved by serologic assays. Virus detection assays for TBE complex viruses are often used to conduct surveillance of tick populations to determine the geographic distribution of virus activity. These data can then be used to target areas for human vaccination. Only one RT-PCR assay is described in which TBE complex consensus primers are designed to amplify all members of the serocomplex (Whitby *et al.*, 1993). The remainder are assays for the specific detection of either TBE or Louping ill viruses (Gaunt *et al.*, 1997; Kreil *et al.*, 1997; Ramelow *et al.*, 1993; Schrader *et al.*, 1999; Wicki *et al.*, 2000) (Table II). The TBE assays have primarily been used for the detection of viral RNA in field collected ticks, looking for focal regions of TBE activity. Little data on the utility of these assays for detecting TBE viral RNA in human clinical specimens (i.e., cerebrospinal fluid) from suspected cases has been documented. One paper reports on the successful detection of TBE from postmortem brain tissue (Tomazic *et al.*, 1997). The TBE complex assay successfully amplified RNA from geographically diverse TBE strains (both Central European and Far Eastern), as well as from Langat, Kyasanur Forest disease, Negishi, Powassan, and Louping ill (Whitby *et al.*, 1993). However, using this system requires additional analysis to identify the amplified DNA (i.e., hybridization with virus-specific probes or nucleic acid sequencing) because all TBE complex viruses are amplified with these primers. The TBE-specific assays described in the literature make use of either a standard two-step or a single-step RT-PCR protocol with detection by size on agarose gel

TABLE II

RT-PCR Assays for Tick-Borne Encephalitis Serocomplex Viruses

Reference	Viral target	Primer location[a]	Amplification format	Detection format	Detection limit[b]	Comments[c]
Ramelow *et al.*, 1993	TBE	5′NC & CAP	2-step RT-PCR	southern blot hybridization	ND	assay succesfully detected TBE-CE in ticks
Whitby *et al.*, 1993	TBE	M & ENV	2-step RT-PCR	agarose	ND	primers detected: TBE-CE, TBE-FE, POW, NEG, LI
Kreil *et al.*, 1997	TBE	M & ENV	2-step RT-PCR	agarose	0.1 pfu	detected TBE in the presence of neutralizing antibody
Schrader *et al.*, 1999	TBE	5′NC	1-step RT-PCR	nested PCR & agarose	20 fg TBE RNA	assay succesfully detected TBE in ticks
Wicki *et al.*, 2000	TBE	NS5	1-step TaqMan RT-PCR	TaqMan	10 RNA copies	assay succesfully detected TBE in ticks
Gaunt *et al.*, 1997	LI	M & ENV	1-step RT-PCR	agarose	ND	detected LI in ticks

[a] Gene abbreviations: 5′NC = 5′non-coding region; C = capsid; M = membrane; ENV = envelope; NS5 = non-structural 5.

[b] Abbreviations: ND = not determined; pfu = plaque-forming units.

[c] Virus abbreviations: TBE-CE = tick-borne encephalitis Central European; TBE-FE = tick-borne encephalitis Far Eastern; POW = Powassan; NEG = Negishi; LI = Louping III.

electrophoresis. A comparison of sensitivity among the various assays is difficult because each one uses different units in calculating sensitivity limits (i.e., copy number, pfu, or quantity of RNA in fg). One TaqMan RT-PCR assay is described for TBE virus (Wicki *et al.*, 2000). Primers were designed with homology to TBE-CE and no data are provided on detection of other strains of TBE virus or other viruses within the serocomplex. One RT-PCR assay is described for the detection of Louping ill virus in collected ticks (Gaunt *et al.*, 1997). The assay demonstrated a sensitivity equivalent to virus isolation in tissue culture and was used successfully to detect Louping ill virus in field-collected ticks.

3. Japanese Encephalitis Serocomplex Viruses

There are a number of viruses in this serologic complex that are associated with human disease, the most prominent include Japanese encephalitis (JE), West Nile (WN), Murray Valley encephalitis (MVE), St. Louis encephalitis (SLE), and Kunjin. In general, viremia during most JE complex virus infection in humans is short lived and rarely detectable by the time clinical manifestations appear. As a result, virus detection assays testing serum (i.e., virus isolation, PCR, or NASBA) are of limited use in diagnosing human infections because negative results are inconclusive. Testing cerebrospinal fluid (CSF) specimens can be more productive than testing serum; in one report, 57% of CSF specimens obtained from WN infected humans tested positive in a TaqMan RT-PCR assay (Lanciotti *et al.*, 2000). In contrast, virus detection assays are most often used for surveillance of virus activity in nature through testing of mosquito vectors and/or associated vertebrate hosts. In many cases, JE complex viruses can be detected in tissues at autopsy by immunohistochemistry or by molecular amplification methods. RT-PCR assays for detecting JE serocomplex viruses are summarized in Table III. Three standard format RT-PCR assays have been developed for the detection of SLE virus, two of which have been compared to virus isolation in tissue culture for detecting SLE virus from mosquito pools (Howe *et al.*, 1992; Lanciotti *et al.*, 2001; Nawrocki *et al.*, 1996). Both demonstrated sensitivity very close to that obtained in tissue culture in testing field collected mosquitoes. JE virus has been successfully detected from laboratory infected mosquitoes, mosquito larvae, and from blood obtained from infected mice in two standard RT-PCR assays, one of which incorporates digoxigenin labeling of the amplified DNA and colormetric detection of the amplified product (Murakami *et al.*, 1994; Paranjpe *et al.*, 1998). Igarashi demonstrated the ability of a JE RT-PCR assay to detect JE in CSF

TABLE III

MOLECULAR AMPLIFICATION ASSAYS FOR JAPANESE ENCEPHALITIS SEROCOMPLEX VIRUSES

Reference	Viral target[a]	Primer location[b]	Amplification format	Detection format	Detection limit[c]	Comments
Howe *et al.*, 1992	SLE	CAP, PrM, & ENV	2-step RT-PCR	agarose	200 RNA copies	tested only 2 SLE strains
Porter *et al.*, 1993	WNV	NS3	2-step RT-PCR	agarose	6.5 pfu	
Murakami *et al.*, 1994	JE	ENV	2-step nested RT-PCR	agarose	3–5 particles (EM)	detected JE in spiked blood
Nawrocki *et al.*, 1996	SLE	ENV	1-step RT-PCR	agarose	ND	tested 1,725 mosquito pools
Paranjpe *et al.*, 1998	JE	ENV	1-step RT-PCR	agarose	1 pg RNA	
Briese *et al.*, 2000	WNV	NS3 & NS5	1-step TaqMan RT-PCR	TaqMan	50–100 RNA copies	detected WNV in CSF specimens
Lanciotti *et al.*, 2000	WNV	ENV & 3′NC	1-step TaqMan RT-PCR	TaqMan	0.1 pfu	detected WNV in CSF, mosquitoes, and birds
Hadfield *et al.*, 2001	WNV	3′NC	1-step TaqMan RT-PCR	TaqMan	1 pfu	
Lanciotti *et al.*, 2001	SLE	PrM/M & ENV	1-step TaqMan RT-PCR	TaqMan	0.1 pfu	North & South American SLE strains detected
Lanciotti *et al.*, 2001	WNV & SLE	PrM/M & ENV	NASBA	ECL & molecular beacons	0.1 pfu	detected WNV in CSF specimens

[a] Virus abbreviations: SLE = St. Louis encephalitis; WNV = West Nile virus; JE = Japanese encephalitis.

[b] Gene abbreviations: C = capsid; PrM = pre-membrane; M = membrane; ENV = envelope; NS3 = non-structural 3; NS5 = non-structural 5; 3′NC = 3′ non-coding region.

[c] Abbreviations: pfu = plaque forming units; EM = electron microscopy; ND = not determined.

samples obtained from acute encephalitis cases in Karachi, Pakistan (Igarashi *et al.*, 1994). The recent discovery of West Nile virus in the United States has stimulated the development of several new RT-PCR assays for the detection of WN virus from a variety of specimen types. Two standard RT-PCR assays and three TaqMan-based assays have been described in the literature (Briese *et al.*, 2000; Hadfield *et al.*, 2001; Lanciotti *et al.*, 2000; Porter *et al.*, 1993). The primary application of these assays has been in detecting WN virus activity in nature by testing bird tissues and mosquito pools. In general the TaqMan assays have proven to be more sensitive than both tissue culture and standard format RT-PCR assays, although a nested RT-PCR has been shown to be as sensitive as the TaqMan systems (Shi *et al.*, 2001). These assays have also been used to detect WN in acute CSF specimens with limited success (Briese *et al.*, 2000; Lanciotti *et al.*, 2000).

4. Dengue Viruses

Molecular amplification assays for the detection of dengue viruses were among the first RT-PCR assays for flaviviruses to be described in the literature. The large number of assays that have been developed for dengue (Table IV) is clearly related to the high incidence of human infection worldwide and the need for a sensitive diagnostic test for virus detection and serotype identification. Virus detection assays for dengue have always been an essential component of human diagnostic testing for dengue infection because, in contrast to other flavivirus infections, dengue viruses generate viremias that are generally higher and of longer duration. In addition, serologic assays, which are the primary diagnostic test for most flavivirus infections, are of limited utility in many (i.e., secondary) dengue virus infections. Therefore virus isolation with serotype identification by immunofluorescence has traditionally been the “gold standard” for dengue virus detection and serotype identification in diagnostic laboratories.

Table IV displays most of the amplification assays that have been developed for dengue virus detection. Dengue assays that described detection of only one serotype or that were minor modifications/applications of previously described assays were not included in Table IV (Chan *et al.*, 1994; Chen *et al.*, 1992; Fung *et al.*, 2000; Maneekarn *et al.*, 1993; Morita *et al.*, 1994; Thayan *et al.*, 1995; Yamada *et al.*, 1999; Vaughan *et al.*, 1999). The standard RT-PCR assays shown in Table IV use either dengue consensus primer pairs to amplify all four serotypes with one primer set (Henchal *et al.*, 1991; Lanciotti *et al.*, 1992; Sudiro *et al.*, 1997; Yenchitsomanus *et al.*, 1996) or four unique

TABLE IV

MOLECULAR AMPLIFICATION ASSAYS FOR DENGUE VIRUSES

Reference	Primer location[a]	Amplification format	Primer format	Detection format	Detection limit[b]	Serum[c]
Deubel *et al.*, 1990	ENV	2-step RT-PCR	4 type-specific primer pairs	southern blot hybridization	1 MIP DEN-2	100% (24)[d]
Morita *et al.*, 1991	ENV & NS2a&b	1-step RT-PCR	4 type-specific primer pairs	dot-blot hybridization	2.5 pfu	ND
Henchal *et al.*, 1991	NS1	2-step RT-PCR	dengue consensus primer pair	dot-blot hybridization	4.4 pfu	80% (50)
Lanciotti *et al.*, 1992	CAP & PrM	1-step RT-PCR	dengue consensus primer pair	semi-nested with type-specific primers	100 RNA copies	96% (93)
Pao *et al.*, 1992	M & ENV	2-step RT-PCR	2 type-specific primer pairs[e]	agarose	<100 pfu	100% (5)
Seah *et al.*, 1995	NS3	2-step RT-PCR	1 consensus primer + 4 type-specific primers	agarose	1.4–3.5 pfu	ND
Yenchitsomanus *et al.*, 1996	ENV & NSI	2-step RT-PCR	dengue consensus primer pair	nested with type-specific primers	0.1 pfu DEN-2	100%(3)
Sudiro *et al.*, 1997 & 1998	3′NC	2-step RT-PCR	3 dengue consensus primers	microtiter plate hybridization	2–20 FFU	97% (39)
Harris *et al.*, 1998[f]	CAP & PrM	1-step RT-PCR	dengue consensus primer pair	agarose	1–50 pfu	ND
Wang *et al.*, 2000	CAP	1-step RT-PCR[g]	dengue consensus primer pair	agarose	ND	ND
Lucia *et al.*, 1994	NS1[h]	in situ RT-PCR	dengue consensus primer pair	immunohistochemistry	ND	ND
Laue *et al.*, 1999	NS5	1-step TaqMan RT-PCR	4 type-specific primer/probe sets	TaqMan	2 RNA copies	80% (25)[i]
Houng *et al.*, 2000	3′NC	2-step TaqMan RT-PCR	DEN-2 type-specific primer/probe set	TaqMan	0.1 pfu	100% (4)
Houng *et al.*, 2001	3′NC	2-step TaqMan RT-PCR	6 primers + 2 consensus probes	TaqMan	0.04 pfu	89% (134)
Wu *et al.*, 2001	3′NC	NASBA	dengue consensus primer pair	NASBA ECL with type-specific probes[j]	0.01–0.1 pfu	99% (67)

(*continues*)

TABLE IV (*continued*)

[a] Gene abbreviations: ENV = envelope; NS2a&b = non-structural 2a & 2b; NS1 = non-structural 1; CAP = capsid; PrM = pre-membrane; M = membrane; NS3 = non-structural 3; 3′NC = 3′ non-coding region.

[b] MIP = mosquito infecting particles; pfu = plaque forming units; FFU = fluorescent focus units.

[c] The percentage displayed is the number of serum samples which tested positive by RT-PCR divided by the number of samples testing positive by virus isolation. The number in parenthesis is the total number of samples tested. ND = not determined

[d] There were nine additional serum samples that tested positive by RT-PCR that were negative by isolation; all from confirmed dengue cases.

[e] The assay was for DEN-1 and DEN-2 only.

[f] Modification of the Lanciotti (1992) protocol combining consensus and type specific primers in a single reaction and using a newly designed DEN-4 primer.

[g] Competitive RT-PCR for quantification of dengue RNA.

[h] Primers originally described by Henchal (1991).

[i] The 25 samples tested were not assayed for virus by isolation; infection with one of the dengue serotypes was confirmed by serological tests.

[j] NASBA-ECL = NASBA amplification followed by hybridization with type-specific capture probes and detection by electrochemiluminescence; see Section III for details.

primer pairs (Deubel *et al.*, 1990; Morita *et al.*, 1991; Pao *et al.*, 1992). In some cases a combination of consensus and type-specific primer pairs is used for the primary amplification (Harris *et al.*, 1998; Seah *et al.*, 1995). Serotype identification is accomplished either by molecular hybridization with serotype-specific probes (Deubel *et al.*, 1990; Henchal *et al.*, 1991; Morita *et al.*, 1994) or by nested PCR with type specific primers to generate unique sized fragments for each of the serotypes (Lanciotti *et al.*, 1992; Seah *et al.*, 1995; Yenchitsomanus *et al.*, 1996). Two assays use an RNA competitor at increasing concentrations during amplification to estimate the amount of viral RNA in the original sample (Sudiro *et al.*, 2001; Wang *et al.*, 2000); however, the TaqMan assays offer a much easier and more accurate approach to quantitation (see subsequent section). One in situ RT-PCR assay is described that offers the ability to detect dengue RNA in paraffin-embedded tissues (Lucia *et al.*, 1994). Finally, two TaqMan assays have recently been described: one uses four serotype-specific primer/probe sets (Laue *et al.*, 1999) while the other uses a combination of consensus and type-specific primers and probes (Houng *et al.*, 2000; Houng *et al.*, 2001). A direct comparison of the detection limits of the various assays using quantitated virus is difficult because of the different units used to quantitate virus. It appears, however, that all of the standard format RT-PCR assays can detect at least approximately 10 pfu of virus. The detection limit of both TaqMan assays exceeds (detecting <0.1 pfu) that of all of the standard RT-PCR amplification systems. The application of the various assays in detecting dengue viral RNA in clinical serum samples was evaluated for most of the assays; however, the number of samples tested varied considerably (3–134 samples tested). When compared to virus isolation, the RT-PCR assays correctly detected and identified dengue virus in serum specimens from 80% to 100% of the time.

IV. NASBA Assays for Flaviviruses

Recently NASBA assays have been developed for SLE, WN, and dengue viruses (Lanciotti and Kerst, 2001; Wu *et al.*, 2001). The SLE and WN assays use NASBA-based amplification with virus-specific primers. Detection of the amplified RNA is accomplished either by ECL detection using the NucliSens reader or detection in real time with fluorescent-labeled molecular beacon probes. ECL and molecular beacon detection displayed approximately the same sensitivity in detecting quantitated SLE and WN virus; however, the molecular

beacon assay took less than 1 hour to complete. These NASBA assays were also directly compared to SLE and WN TaqMan and standard format RT-PCR assays. In general the NASBA assays displayed the same sensitivity and specificity as TaqMan assays when testing quantitated virus or diagnostic specimens. In all comparisons, NASBA and TaqMan were more sensitive than standard format RT-PCR assays. The dengue NASBA assay (Table IV) uses two consensus amplification primers located in the 3′ non-coding region of the genome. Detection and serotype identification is achieved through the use of four serotype-specific capture probes in the ECL detection format. This assay was able to detect 0.01 pfu of each of the four serotypes spiked into normal human serum. In addition, the assay detected and correctly identified the dengue virus serotype in 66 of 67 viremic serum specimens (99%).

V. Conclusion

The development of molecular amplification assays for flaviviruses, using either RT-PCR–based methods or NASBA technology, has enabled the rapid detection of most of the medically important flaviviruses. In general, these amplification technologies offer sensitivity that is similar to, or in some cases exceeds isolation in cell culture. The primary benefit of these assays is that they offer very rapid turnaround time when compared to virus isolation; results in some cases can be obtained in less than 1 hour compared to over 1 week for isolation in tissue culture. The ability to rapidly detect flaviviruses could have a positive impact on preventing human disease because laboratory results could direct timely public health interventions (i.e., vaccination, pesticide application, etc.).

Initially, molecular amplification assays were limited to laboratories with expertise in the area of molecular biology. However, the development of a number of commercially available kits for nucleic acid extraction and amplification has led to more widespread use of these assays in many laboratories. The recent development of TaqMan and NASBA assays that use instruments for data collection and interpretation will greatly improve sensitivity of these assays and also lead to better standardization of testing among laboratories. These approaches can also be automated, which will further improve reproducibility and standardization.

However, even though there has been rapid growth and improvement in the area of molecular amplification assays, it is still important

for reference laboratories to conduct virus isolation in tissue culture. This is necessary because in some cases molecular assays may fail to detect strain variants of known viruses (due to primer mismatches), and most likely these assays would not detect new flaviviruses. Second, virus isolation should be conducted so that viruses can be catalogued and subjected to further characterization and analysis. For these reasons, isolation in tissue culture should remain the "gold standard" test of reference laboratories if virus discovery and ongoing research are to remain a priority.

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VIRUS EPIDEMIOLOGY, ECOLOGY, AND EMERGENCE

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ADVANCES IN VIRUS RESEARCH, VOL 61

JAPANESE ENCEPHALITIS

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I. History

Japanese encephalitis (JE) is an acute mosquito-borne viral disease of people, horses, swine, and other domestic animals caused by a flavivirus closely related to Murray Valley encephalitis. A synonym is Japanese B encephalitis, to distinguish the syndrome from Von Economo, or type A, encephalitis.

Summer-fall encephalitis outbreaks suggestive of Japanese encephalitis (JE) were recorded in Japan as early as 1871, the largest, in 1924, involving more than 6000 cases, 60% of them fatal (Hiroyama, 1962). In 1934, Hayashi succeeded in producing experimental encephalitis with an isolate of this virus in monkeys (Hayashi, 1934; Inada, 1937). Around 1935, many groups were successful in demonstrating neutralizing antibody in recovered patients that protected mice against challenge with JE, establishing JEV as the causal agent

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of the encephalitis (Kasahara *et al.*, 1936; Kawamura *et al.*, 1936; Taniguchi *et al.*, 1936). This discovery enabled serologic confirmation of encephalitis cases occurring elsewhere in the region demonstrating the geographic distribution of JE, including a cluster of cases that occurred in Beijing in 1934 and 1935 (Kuttner and T'sun, 1936). With the recovery of strains of St. Louis encephalitis (SLE) in 1933 and West Nile viruses in 1940, a distinct antigenic group was recognized (Smithburn, 1942). The virus initially was called B encephalitis in deference to Von Economo's type A encephalitis, which had different clinical and epidemiologic characteristics (the modifying "B" has since fallen into disuse). In 1938, Hammon *et al.* provided evidence supporting the mosquito-borne mode of JE transmission by isolating JE virus from *Culex tritaeniorhynchus* mosquitoes (Hammon *et al.*, 1949). Subsequent field studies later established the role of aquatic birds and pigs in the viral enzootic cycle (Scherer and Buescher, 1959). Viruses isolated from human cases in Japan in 1935 and in Beijing in 1949 provided the prototype Nakayama, Beijing, and P3 strains, respectively, that are used in vaccine production today. During the first half of the 20th century, JE was recognized principally in temperate areas of Japan, Korea, and China. Over the past three decades, increasing epidemic activity has been reported from much of Southeast Asia, India, and Sri Lanka.

II. Clinical Description

The incubation period is 5 to 15 days and typically progresses through four stages: a prodromal illness (2–3 days), an acute stage (3–4 days), a subacute stage (7–10 days), and convalescence (4–7 weeks). A proportion of patients make a rapid spontaneous recovery after completing their clinical course.

Typically patients with JE present after a few days of nonspecific febrile illness, which may include coryza, diarrhea, and rigors (Solomon, 1997). This is followed by headache, vomiting, and a reduced level of consciousness, often heralded by a convulsion. Both the presenting symptoms and the clinical course of JE can vary widely with extensive neurologic symptoms. Although milder clinical presentations, such as aseptic meningitis, febrile convulsions, or a respiratory-like febrile illness have been recognized, the principal clinical manifestation of illness is encephalitis (Endy and Nisalak, 2002; Halstead and Grosz, 1962; Solomon and Vaughn, 2002). Due to its extensive symptoms and neurologic involvement, JE may best be described as a meningoencephalomyelitis with some patients displaying meningeal signs while

5–20% of comatose patients demonstrate flaccid weakness indicative of lower motor neuron involvement (Endy and Nisalak, 2002; Solomon and Vaughn, 2002).

The acute stage may be heralded by a high fever. Grand mal seizures, usually generalized and major motor in type, are seen in 10–24% of children but are less common in adults. The classic presentation of JE includes a dull flat mask-like facies with wide unblinking eyes, tremor, generalized hypertonia, and cogwheel rigidity (Kumar *et al.*, 1990; Solomon *et al.*, 2000). Occasionally respiratory muscle paralysis may be the presenting feature (Tzeng, 1989). In some patients, particularly older children and adults, abnormal behavior may be the only presenting feature, resulting in an initial diagnosis of mental illness. This type of presentation was seen during the Korean War when some American servicemen with Japanese encephalitis were initially diagnosed as having "war neurosis" (Lincoln and Siverson, 1952).

Patients with JE may also present with a poliomyelitis-like acute flaccid paralysis (Solomon *et al.*, 1998). After a short febrile illness, there may be the rapid onset of flaccid paralysis in one or more limbs, despite a normal level of consciousness. Weakness occurs more often in the legs than the arms, and is usually asymmetric. Encephalitis subsequently developed in 30% of such patients, with reduced level of consciousness and upper motor neuron signs, but in most, acute flaccid paralysis was the only feature. In these patients at follow-up 1–2 years later, there was persistent weakness and marked wasting in the affected limbs. Electrophysiologic studies confirmed anterior horn cell damage and magnetic resonance imaging (MRI) of the spinal cord showed abnormal signal intensity on T2-weighted images (Kumar *et al.*, 1997).

The subacute and convalescent stages are characterized by varying degrees of neurologic involvement. Some practitioners have observed that neurologic signs, such as focal seizures or asymmetric paralysis, differentiate JE from encephalitis of other etiologies (Rao, 2002). Parkinsonian-like non-intention tremor and cogwheel rigidity are seen less frequently. Other extrapyramidal features include head nodding and pill rolling movements, opsoclonus, myoclonus, choreoathetosis, and bizarre facial grimacing and lip smacking (Kumar *et al.*, 1990; Misra and Kalita, 1997; Solomon *et al.*, 2000). Upper motor neuron facial nerve palsies occur in approximately 10% of children and may be subtle or intermittent. Frequently there are signs of upper motor neuron involvement and meningeal irritation. Particularly characteristic are rapidly changing central nervous system signs (e.g., hyperreflexia followed by hyporeflexia or plantar responses that change). The

sensory state of patients may vary from confusion, disorientation, delirium, or somnolence, progressing to coma. Poor prognostic signs include changes of respiratory patterns, flexor and extensor posturing, and abnormalities of the papillary and oculocephalic reflexes and may indicate encephalitis in the brainstem (Hoke *et al.*, 1992; Kumar *et al.*, 1990; Libraty *et al.*, 2002; Solomon *et al.*, 2000, 2002). A substantial proportion of patients become totally unresponsive and require ventilatory assistance. Generalized weakness and changes in tone, focal motor deficits—including paresis, hemiplegia, or tetraplegia; cranial nerve palsies (especially central facial palsy); and abnormal reflexes—also may be present. Signs of extrapyramidal involvement, including tremor, mask-like facies, rigidity, and choreoathetoid movements, are characteristic of JE, but these signs may be obscured initially by generalized weakness (Fig. 1). Sensory disturbances are seen less frequently. Central hyperpnea, hypertension, pulmonary edema, and urinary retention also may complicate the illness.

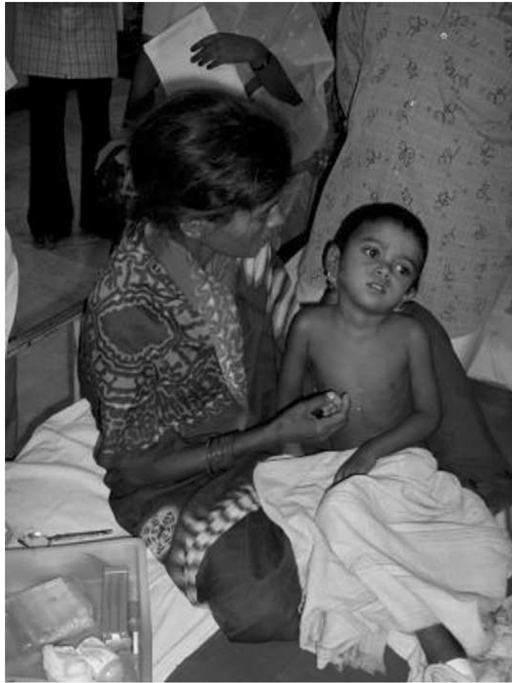


FIG 1. Acute stage of Japanese encephalitis in a 7-year-old Indian girl. Mother is supporting child in sitting position. Adoni, India, November 2002. (See Color Insert.)

Although symptoms suggest elevated intracranial pressure in many cases, papilledema and other signs of increased intracranial pressure are seen only rarely.

Clinical laboratory examination discloses a moderate peripheral leukocytosis with neutrophilia and mild anemia. Polyurea with hyponatremia reflecting inappropriate antidiuretic hormone (ADH) secretion is a frequent complication. Cerebrospinal fluid (CSF) pressure is increased in approximately 50% of patients. High pressures (>250 mm) are associated with poor prognosis (Solomon *et al.*, 2000). Pleocytosis ranges from a few to several hundred cells per cubic millimeter, with a lymphocytic predominance; neutrophils may predominate in early samples. CSF protein is moderately elevated in approximately 50% of cases. Reduced levels of CSF monoamine (homovanillic and 5-hydroxyindoleacetic acids) have been found in the acute phase of illness and in recovery, but these reductions have not correlated consistently with clinical parkinsonism (Kusuhara *et al.*, 1996). Previous dengue infection conveys some protection against severe JE. The ability to mount an early and vigorous JEV antibody response is associated with a better outcome from acute JE. An initial serum anti-JEV IgM <150 U, low levels of serum and CSF IgG antibody against JEV and a serologic response consistent with primary flavivirus infection were significantly associated with death as an outcome of infection (Libraty *et al.*, 2002).

Computed tomography (CT) scans and MRI reveal low-density areas and abnormal signal intensities, respectively, in the thalamus, basal ganglia, pons, and putamen. Acute changes in the thalamus may be a helpful differentiating feature. MRI abnormalities may be seen in the spinal cord, underscoring that JE is an encephalomyelitis. In JE, compared with encephalitis cases resulting from other causes, T2-weighted MRI more frequently disclose bilateral thalamic high-intensity lesions representing hemorrhages, and single photon-emission CT more often shows increased activity in the thalami and putamen (Misra *et al.*, 1994; Shoji *et al.*, 1990). Thalamic lesions of mixed intensity may also be seen on T1- and T2-weighted images, suggesting hemorrhage (Kumar *et al.*, 1997; Misra and Kalita, 1997). Electromyographic changes reflecting anterior horn cell degeneration are detected, especially in patients with clinical wasting; however, abnormalities in somatosensory-evoked potentials are rare, which is consistent with the infrequency of clinical sensory deficits. Delays in central motor conductance time reflect widespread involvement of white matter, thalamus, brainstem, and spinal cord. Single positron emission tomography studies carried out acutely may show hyperperfusion in

the thalamus and putamen (Kimura *et al.*, 1997). Electroencephalography (EEG) tracings typically show theta and delta coma, burst suppression, epileptiform activity, and occasionally alpha coma (Misra *et al.*, 1994). Diffuse slowing may be useful in distinguishing JE from herpes simplex encephalitis in which changes are characteristically frontotemporal (Misra and Kalita, 1998). Imaging and neurophysiologic abnormalities indicative of thalamic damage correlate with several of the clinical manifestations typifying the acute phase of illness.

A range of 5% to greater than 50% of cases are fatal, averaging around 30%, with some deaths occurring after a brief prodrome and fulminant course lasting a few days and others occurring after a more protracted course of persistent coma. Young children (<10 years) are more likely to die than older individuals, and if they survive are more likely to have residual neurologic deficits. Overall, approximately one third of surviving patients exhibit serious residual neurologic disability with a larger proportion demonstrating lesser sequellae (Kumar *et al.*, 1993; Pieper and Kurland, 1958; Schneider *et al.*, 1974; Simpson and Meiklejohn, 1947). Principal sequellae include memory loss, impaired cognition, behavioral disturbances, convulsions, motor weakness or paralysis, and abnormalities of tone and coordination. In children, motor abnormalities frequently improve or eventually resolve, but behavioral changes and psychological deficits have been detected 2 to 5 years after recovery in up to 75% of pediatric cases; EEG abnormalities also may persist in the absence of detectable clinical signs (Edelman *et al.*, 1975). Evidence of previous dengue immunity is associated with better outcome.

Poor prognosis has been associated with a short prodromal interval, clinical presentation in deep obtundation, respiratory dysfunction, prolonged fever, focal presentation, status epilepticus, the presence of extrapyramidal signs, or pathologic reflexes (Burke *et al.*, 1985a, 1985b; Kumar *et al.*, 1994; Ravi *et al.*, 1997). In some locations concurrent neurocysticercosis has been reported in more than one third of JE cases, with evidence of increased mortality in co-infected patients (Desai *et al.*, 1997), suggesting that parasitic infection could enhance neuroinvasion by impairing the integrity of the blood-brain barrier.

A few observers suggest that infection may fail to clear in certain individuals, with clinical relapse observed several months after resolution of the acute illness (Sharma *et al.*, 1991). In some of these cases virus was recovered from persistently infected peripheral lymphocytes in the presence of circulating antibody. Other patients studied weeks or months after recovery had asymptomatic viremias, virus or viral

antigen in CSF and immunoglobulin M (IgM) JE antibodies in the CSF (Ravi *et al.*, 1993). The question of chronic or recurrent JE infections requires further study.

No specific therapy is available, but as evidenced by causes of death in a large Indian study, appropriate supportive treatment should significantly reduce morbidity and mortality (Fig. 2). Care should be focused on the major preventable causes of death: aspiration, status epilepticus, increased intracranial tension, hypoglycaemia, hyperpyrexia, asphyxia, hypotension, electrolyte imbalance, and secondary infections (Rao, 2002). It is important to keep the airway clear, provide proper nutrition and hydration, manage seizures, and avoid bed sores and secondary infections. Patients are frequently agitated and should be kept away from over stimulating environments (Rao, 2002). Mannitol and other modalities to control intracerebral pressure often are needed. However, in a controlled trial, dexamethasone therapy did not improve outcome (Hoke *et al.*, 1992). Trihexyphenidyl hydrochloride and central dopamine agonists have been used to treat acute extrapyramidal symptoms (Huy *et al.*, 1994). Neutralizing murine monoclonal anti-JE antibodies, developed in China, have been reported to improve clinical outcome in small controlled clinical trials, and licensure in that country has been sought (Ma *et al.*, 1992). Experimental studies in mice and monkeys also suggest the potentially beneficial

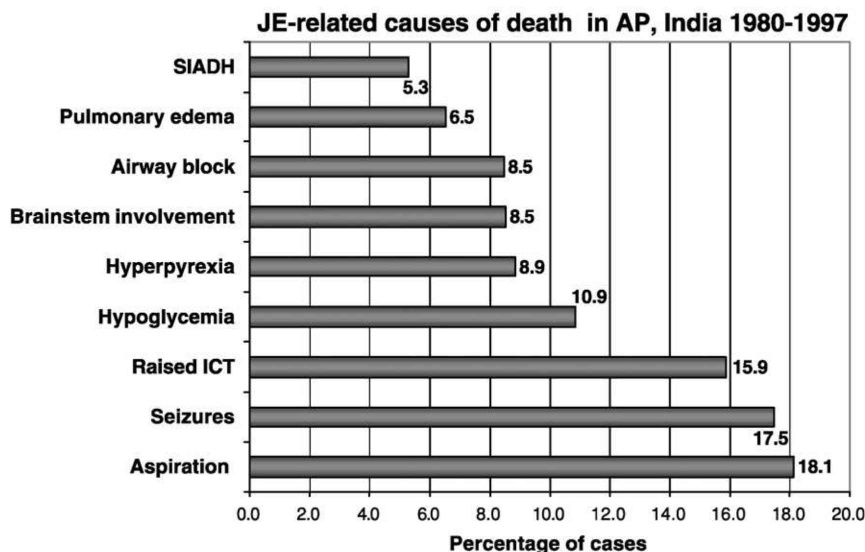


FIG 2. Causes of death in 12,506 JE patients, Andhra Pradesh, India (Rao, 2002).

effect of interferon, and in an uncontrolled series of 14 patients treated with recombinant interferon-alpha, 13 survived (Harinasuta *et al.*, 1985). However, in a randomized trial of interferon alpha treatment for acute JE encephalitis, there was no benefit associated with the drug (Solomon *et al.*, 2003). A number of antiviral compounds, including ribavirin, exhibit activity in vitro but have not been evaluated clinically. *In vivo*, non-steroidal anti-inflammatory agents (NSAIAS) have demonstrated antiviral activity inhibiting viral replication and cellular apoptosis. This effect may deserve further evaluation in a clinical setting (Chen *et al.*, 2002; Liao *et al.*, 2001).

III. Congenital Infection

JE was associated with adverse events in human pregnancy in a series of outbreaks in Uttar Pradesh, India (Chaturvedi *et al.*, 1980; Mathur *et al.*, 1985). JE infections were documented in nine pregnant women: four of the women were infected with JE in the first or second trimester, all of whom miscarried; of these four, virus was recovered from products of conception in two cases. In five women who acquired the illness in the third trimester, no adverse outcomes of pregnancy were observed; however, JE virus–specific IgM was not measured in the infants, and it is unknown whether they were congenitally infected. Experimental data also suggest that the risk of congenital infection may be related to gestational age. Human placental organ cultures, obtained from medically terminated pregnancies at 8 to 12 weeks' gestation, supported JE viral replication, but tissues from full-term pregnancies were resistant to infection (Bhonde and Wagh, 1985). It is not known whether congenital infection can cause fetal malformation or if asymptomatic infection during pregnancy leads to fetal infection or adverse outcome.

IV. Animal Infections

In Japan, before 1948, epizootics of encephalitis in horses tended to coincide with human epidemics. The incidence of JE in horses was 10 to 50 per 100,000 with case fatality rates of approximately 50% (Matsuda, 1962). Swine develop inapparent infections and circulate virus in blood for 3–4 days. Unlike horses, pigs are believed to be important in the transmission of JE to humans, serving as the primary amplifying host. In some areas sentinel pigs are used to predict the risk of human outbreaks. Infection in gestating swine may be transmitted

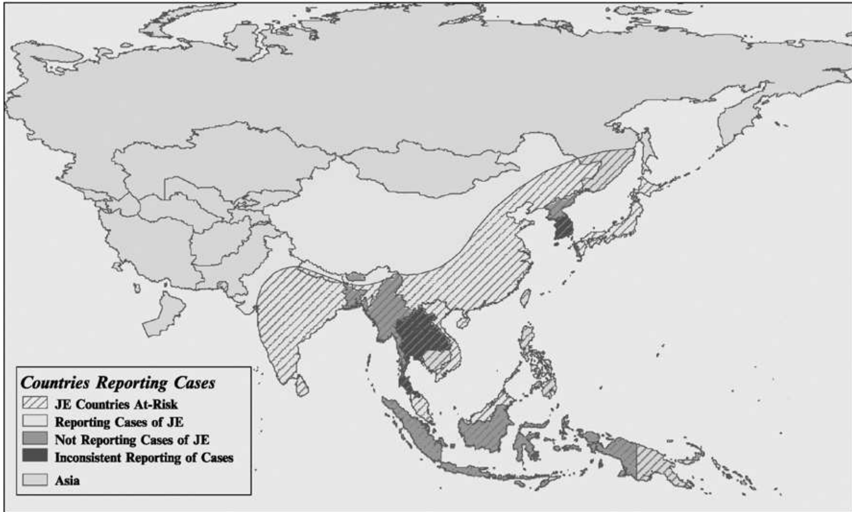
to their fetuses in utero, causing stillbirths or farrowing of weak pigs. Up to 42% may succumb during the first week of life (Burns, 1950; Shimizu and Kawakami, 1949). In peak transmission periods as many as 33% of sows have aborted their litters (Takashima *et al.*, 1988). As a result, in Japan, pigs are bred early in the year to minimize loss of fetuses due to JE infection in pregnant sows. This practice results in the birth of piglets at a time when they are available to support transmission during the peak months of July through September. Since 1972, live-attenuated vaccines have been licensed in Japan for use in horses and pigs (Fujisaki *et al.*, 1975; Goto, 1976). Although use of pig vaccination has demonstrated a decrease in transmission to humans, it has not been pursued as a public health policy due to high turnover in the pig population and the interference of maternal antibody (Igarashi, 2002).

Bird-mosquito cycles are thought to be important in maintaining and amplifying JE virus in the environment (Buescher *et al.*, 1959b; Rodrigues *et al.*, 1981; Scherer *et al.*, 1959; Soman *et al.*, 1977;). Viremia frequently accompanies infection of both wild and domestic birds. The amount of virus circulating in experimentally infected birds is adequate to infect vector mosquito species. High rates of JE antibodies are found in adults of many species of birds (Rodrigues *et al.*, 1981). These antibodies are transferred through the egg to chicks, providing protection against viremic infection for approximately the first 5 weeks of life (Scherer *et al.*, 1959).

V. Geographic Distribution

Countries with proven epidemics of JE include China, maritime Siberia, Korea, Japan (including Okinawa), Taiwan, Guam, Saipan, Vietnam, Cambodia, Thailand, India, Nepal, and Sri Lanka. In these countries JE is the leading recognized cause of childhood encephalitis (Hammon *et al.*, 1958; Vaughn and Hoke, 1992) (Fig. 3). Sporadic cases have been reported from the Philippines, Malaysia, Singapore, Indonesia, Myanmar, and Bangladesh. Within the Southeast Asia Region only 40% of countries report any JE data and within the Western Pacific Region, data is reported inconsistently (Igarashi, 1992; Umenai *et al.*, 1985) In these two regions, in the past decade as many as 35,000 cases and 10,000 deaths have been reported annually (Fig. 4). In several countries, the disease is not under systematic surveillance, and official reports undoubtedly underestimate the true number of cases. Although the disease is presently transmitted only in Asia,

Countries Officially Reporting Japanese Encephalitis to WHO



Source: United States Centers for Disease Control and Prevention
World Health Organization

This map was compiled by the Children's Vaccine Program at PATH using data believed to be accurate. However, a degree of error is inherent in all maps. No attempt has been made in either the design or production of the maps to define the limits or jurisdiction of any federal, state, or local government.

FIG 3. Countries reporting Japanese encephalitis cases to the World Health Organization. (See Color Insert.)

because the region contains more than 3 billion people and 60% of the world's population, regional JE-associated morbidity may exceed worldwide morbidity from herpes encephalitis, the latter estimated at 5 per 1 million per year, or approximately 30,000 cases worldwide (Tsai *et al.*, 1999). With the near eradication of poliomyelitis, JE now is the continent's leading cause of childhood viral neurologic infection and disability. By any standard, JE is a major public health problem and one that can be controlled by effective vaccines.

Before the introduction of vaccine, China accounted for the majority of cases; between 1965 and 1975, more than 1 million cases were reported; 175,000 in 1971 alone. In Japan, several thousand cases were reported annually from the end of World War II. In Korea, 5616 cases and 2729 deaths were recorded in 1949, epidemics continuing every 2 or 3 years and culminating in 6897 cases in 1958. In Taiwan, 1958 and 1951 were epidemic years with 1800 and 704 cases, respectively (Green *et al.*, 1963). Since the end of World War II, sporadic JE cases have been reported from Singapore and Malaysia (Cardosa *et al.*, 1995; Pond *et al.*, 1954).

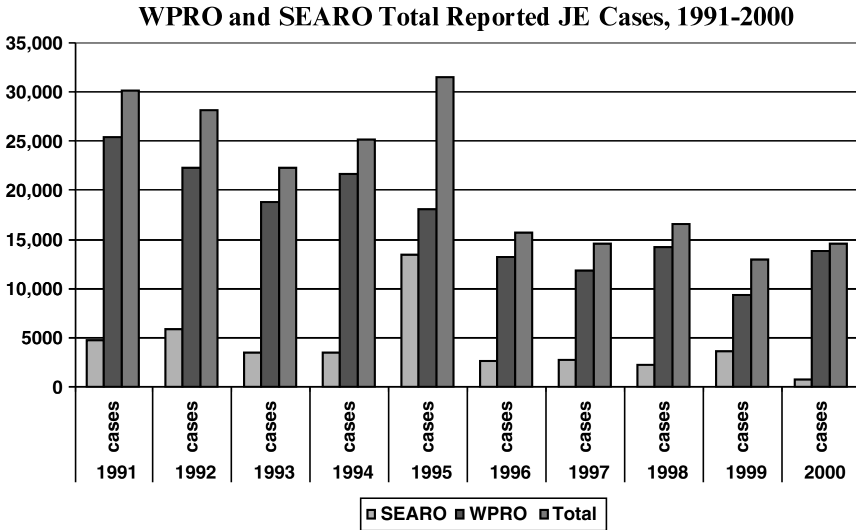


FIG 4. Japanese encephalitis cases reported to the Western Pacific and South-East Asian offices of the World Health Organization, 1991–2000.

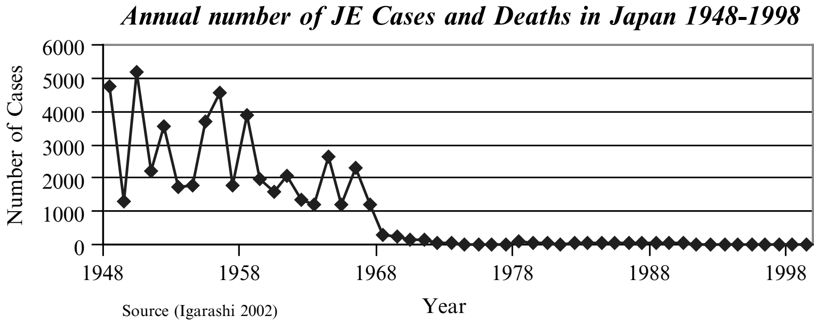


FIG 5. JE cases reported annually in Japan, 1948–1998 (Igarashi, 2002).

Introduction of widespread vaccination in the late 1960s led to the near elimination of JE in economically advanced Asian countries such as Japan, Korea, and Taiwan (Fig. 5). JE vaccines have greatly lessened the disease burden in China. At the same time, investments in new crops and animal husbandry in Southeast and South Asia appear to have increased the geographic spread and/or rates of transmission of JE to human beings (Fig. 6). Sporadic viral encephalitis cases had been noted in northern Thailand for some decades, but in

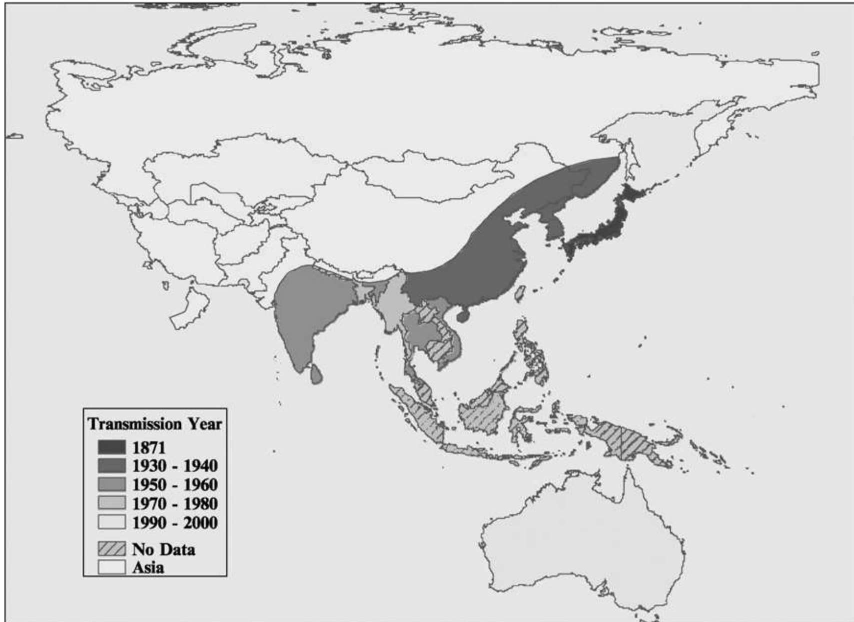


FIG 6. Year of earliest reports of JE outbreaks in Asia. Based on published data and reports to the World Health Organization. (See Color Insert.)

1969, there were 685 cases reported from the Chiang Mai Valley (Grossman *et al.*, 1973). Since then, there have been thousands of cases and hundreds of deaths annually in north Thailand with JE recognized as a leading cause of childhood mortality and disability (Chunsuttiwat and Warachit, 1995). In 1974, the first of several epidemics was recorded in the Shan state of Myanmar (Burma) adjacent to the Chiang Mai Valley (Ming *et al.*, 1977). In Vietnam, with the reinstatement of notification in 1979, several thousand JE cases have been reported annually, and the disease is recognized as a public health problem in the densely populated deltas of the Mekong and Red Rivers (Nguyen and Nguyen, 1995). Incidence rates exceeding 20 per 100,000 have been reported from areas of the northern delta near Hanoi. Clinically confirmed cases have been reported from Cambodia (Chhour *et al.*, 2002; Srey *et al.*, 2002). Only sporadic cases have been documented in the Philippines, Indonesia and Sarawak, Malaysia and in

tourists visiting Bali (Barzaga, 1989; Macdonald *et al.*, 1989; Simpson *et al.*, 1970; Sinniah, 1989). JE may circulate at a level of endemicity that results in case levels that do not excite detection. A recent prospective study on Bali has shown that 40–50% of hospitalized encephalitis cases, mostly children, are caused by JE (Xu, personal communication).

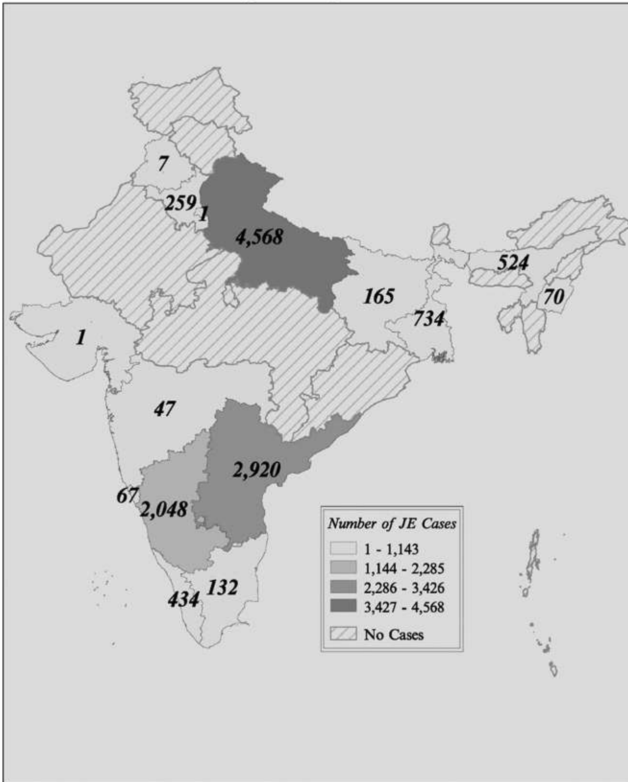
JE transmission was first recognized in Southwest Asia in 1948 in Sri Lanka (Peiris *et al.*, 1992). Sporadic cases and later epidemics were recognized in India around Vellore (Carey *et al.*, 1969). Disease was apparently limited to South India until 1973, when epidemics were reported in Burdwan and Bankura districts of West Bengal and later in Bihar and Uttar Pradesh. Subsequently, large outbreaks of JE, often involving adults, were reported from various Indian states, and now the disease is hyperendemic in northern India and southern Nepal, central India (Andra Pradesh), and southern India (Goa, Karnataka, and Tamil Nadu) (Fig. 7). The question is whether these regional increases in attack rates reflect improved public health surveillance or a real change in viral ecology (Cardosa *et al.*, 1995). One clue to answer this question is found in the age-groups affected by JE. In areas where the disease is newly introduced, JE cases are distributed in all age-groups, as seen in Nepal (Fig. 8). However, in areas where virus may be more enzootically established, such as Vietnam and India, there is almost 100% seroconversion by adulthood and cases are seen primarily in children (Figs. 9 and 10).

VI. Geographic Variation in Virus Strains

On the basis of cross-neutralization, the Flavivirus genus of the Flaviviridae are divided into eight serocomplexes, including the JE serocomplex, which includes West Nile, Kunjin, Murray Valley, and St. Louis encephalitis viruses (Holbrook and Barrett, 2002). The antigenic relationships of JE viruses isolated from different geographic regions and various time periods have been compared by using polyclonal and monoclonal antibodies and divided into at least five antigenic subgroups (Hasegawa *et al.*, 1994; Kedarnath *et al.*, 1986; Kobayashi *et al.*, 1984).

Two-dimensional gel electrophoresis of T1 ribonuclease (RNase)-digested virion RNA and genomic sequencing provide phylogenetic methods to relate JE strains (Chen *et al.*, 1990; Hasegawa *et al.*, 1994; Poidinger *et al.*, 1996). The molecular phylogeny of JE viruses, based on the 240-base nucleotide sequence of the viral the prM region,

Number of Reported JE Cases in States of India from 1997-2001



Source: Personal Communication, P.Nagabhushana Rao, Government of Andhra Pradesh

This map was compiled by the Children's Vaccine Program atPATH using data believed to be accurate. However, a degree of error is in herent in all maps. No attempt has been made in either the design or production of the maps to define the limits or jurisdiction of any federal, state, or local government.

FIG 7. Distribution of JE in India. WHO reports. (See Color Insert.)

divides JE isolates into five distinct genotypes, with a maximum divergence of 21% among the isolates (Chen *et al.*, 1992; Chung *et al.*, 1996; Huong *et al.*, 1993; Ma *et al.*, 1996; Uchil and Satchidanandam, 2001) (Fig. 11). Some workers find only four genotypes, classifying the Muar strain of JE (genotype V) as belonging to genotype III or, based upon amino acid sequences, as a JE complex virus distinct from JE (Holbrook and Barrett, 2002).

The first genotype (I) comprises isolates from northern Thailand, Cambodia, and Korea and a second (II) comprises isolates from southern

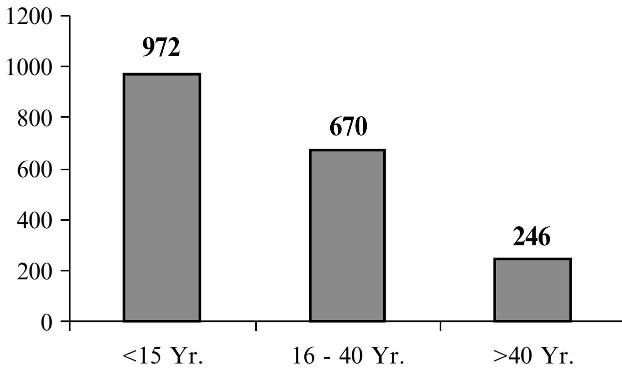


FIG 8. Distribution of JE cases by age-group, Nepal, 2001. WHO reports.

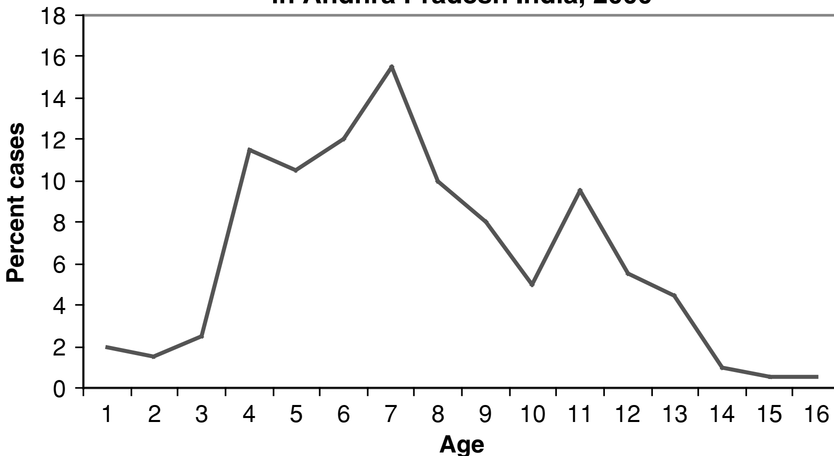


FIG 9. Distribution of JE cases by age, Andhra Pradesh, India, 2001. Andhra Pradesh government reports.

Thailand, Malaysia, Sarawak, Australia, and Indonesia. The largest genotype (III) consists of viruses from Japan, Okinawa, China, Taiwan, Vietnam, the Philippines, Sri Lanka, India, and Nepal. Five Indonesian isolates—two from Java, two from Bali, and one from Flores—similar to each other and distinct from other Indonesian isolates, form the

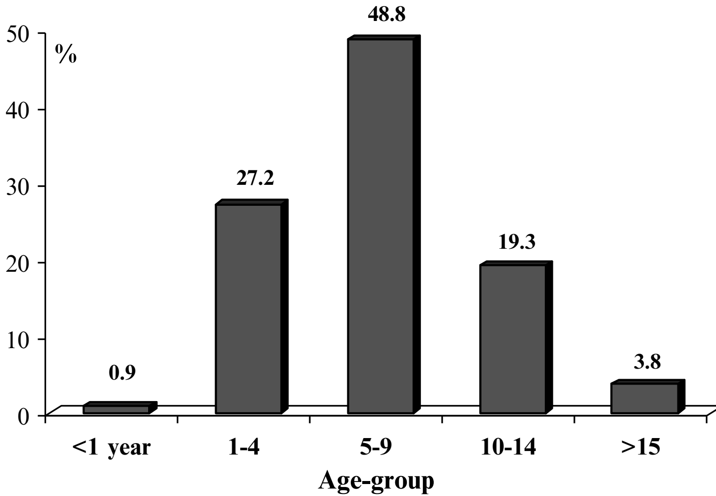


FIG 10. Distribution of JE cases by age-group, Vietnam, 1996–2001. WHO reports.

fourth (IV) genotype. And, a single strain from Singapore comprises a possible fifth genotype (V) (Uchil and Satchidanandam, 2001).

Comparison of the E protein genes of genotype III revealed that they can be further delineated into four subgroups. In 1995, an outbreak of JE occurred on the island of Badu in the Torres Strait between northern Queensland, Australia, and Papua New Guinea (Williams *et al.*, 2000). These strains clustered with genotype group II viruses from Indonesia and Malaysia. However, phylogenetic analysis of full-length genomes showed the Torres Strain FU to be most closely related to a genotype I strain from Korea. However, amino acid analysis did not confirm this relationship (Holbrook and Barrett, 2002). In general, it appears that neither temporal nor geographic boundaries correlate with virus migration or genetic drift of JE viruses. Great care should be taken in constructing phylogenetic trees based on partial nucleotide sequences in the gene databases as many of these strains may be laboratory variants.

Co-circulation of multiple genotypes has been observed in Thailand and Indonesia. JE virus isolates from the same region but from different years show a high degree of nucleotide similarity. Sixteen Vietnamese and 23 Okinawan strains of JE virus isolated between

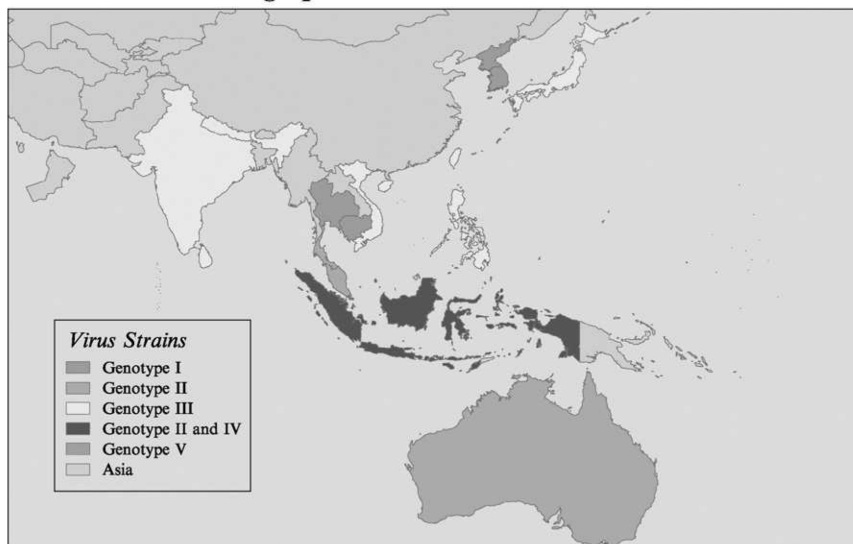


FIG 11. Distribution of JE virus genotypes in Asia (Uchil and Satchidanandam, 2001). (See Color Insert.)

1964 and 1988 and between 1968 and 1992 differed by only 3.2% and 4%, respectively (Huong *et al.*, 1993; Ma *et al.*, 1996). However, viruses from the same region were distinguishable chronologically, before and after 1986 in Okinawa and before and after 1975 in Vietnam. Genetic drift appears to be the main mechanism by which JE virus continuously evolves in nature, although novel viral introductions have been documented, indicating the potential for genotypic displacement (Uchil and Satchidanandam, 2001).

Despite the circulation of multiple genotypes, a genetic analysis of 92 strains in the Gene Bank has shown that the inter-genotype diversity is much less than that observed across poliovirus or dengue serotypes, leading to the conclusion that JE circulates as a single serotype (Tsarev *et al.*, 2000). This has profound implications for the protective efficacy of JE vaccines. Vaccines made from a single genotype should provide protection against infection with strains from all other genotypes. Indeed, a controlled field trial of JE vaccine prepared from a genotype III virus was conducted in a region (Thailand), where a

heterologous genotype was predominant and showed a high degree of efficacy (Hoke *et al.*, 1988).

VII. Recent Changes in Geographic Range

In addition to the apparent spread of amplification of JE in India, Nepal, Southeast Asia, and Bali, Indonesia, described above, JE outbreaks correlate with agricultural development and intensive rice cultivation supported by irrigation schemes (Service, 1991). Hyperendemic transmission of malaria and JE were documented to have followed deforestation and development in the Mahaweli River Valley and Terai in Sri Lanka and southern Nepal, respectively (Peiris *et al.*, 1992, 1993; Service, 1991). The potential for spread of JE is being watched closely in Irian Jaya, Indonesia, the irrigated Thar desert of Rajasthan, and other places under development where conditions receptive to viral transmission and amplification have recently been created (Fig. 11). Recently, JE has been shown to occur as far west as the Indus valley in Pakistan (Igarashi *et al.*, 1994). JE activity has been noted in Penang, Malaysia, and Bali, Indonesia (Cardosa *et al.*, 1991, 1995). A notable increase in cases has been noted in central India (Andrha Pradesh) and southern India (Goa, Karnataka, and Tamil Nadu).

Recent novel introductions leading to outbreaks on Saipan and the Torres Strait islands between New Guinea and northern Australia, and a sporadic case on the Cape York peninsula of mainland Australia, illustrate the potential for JE virus to be transferred over significant distances, possibly by viremic migratory birds or by windblown mosquitoes (Hanna *et al.*, 1996; Paul *et al.*, 1993) (Fig. 6).

VIII. Incidence and Prevalence

Incidence of JE has been shown to vary widely. Where the disease has been closely studied in populations undergoing vaccination trials, incidence has been shown to be high in the range of 2.5–1.8 per 10,000 annually (Tsai, 2000). Extrapolations from routinely reported surveillance data result in lower incidence levels. Due to the differing exposure age, specific incidence rates have also been calculated. Data from Thailand has shown a range of 5–10 per 100,000 under the age of 14 with an incidence of less than 2 per 100,000 in those older than 25 years (Endy and Nisalak, 2002; Solomon and Mallewa, 2001). In

enzootic areas of Asia almost 100% of adults circulate antibody. With symptomatic to asymptomatic case ratios averaging 1:300 to 1:500 in Asia, it would follow that annual JE infection rates must be quite high. Prevalence of disease would need to account for the ongoing disability rates due to JE infections in affected populations. This has not been well studied but would enhance data that supports the cost effectiveness of JE prevention through immunization.

A. *Travellers and Expatriates*

Although JE vaccine is used principally in more economically advantaged countries in Asia to protect residents, the vaccine also is marketed in developed countries for travelers to Asia, expatriates, and military personnel. (For the purposes of this discussion, *expatriates* are defined as non-indigenous residents through a transmission season.) Sporadic cases have been reported in travelers from North America, Europe, Russia, Israel, and Australia and, paradoxically, in Japanese and Taiwanese tourists to other endemic areas of Asia (Buhl *et al.*, 1996; McDonald *et al.*, 1989; Rose *et al.*, 1983).

The risk for acquiring JE during travel is highly variable and depends on the destination and season of travel and the activities of the individual. Although travelers who remain in rural areas for extended periods are at greatest risk, well-publicized and documented cases have been reported in travelers with brief itineraries in resorts or urban locations. For example, in 1996, three cases were reported among travelers to Bali, perhaps a unique situation that may reflect the proximity of local tourist hotels and beaches to areas with pig rearing.

No systematically collected data on cases in travelers are available. Informal surveillance of diagnostic laboratories suggests that risk is extremely low. Transportation statistics indicate that 2 to 3 million U.S. citizens travel by air to Asia each year; however, these figures overestimate the population at risk, because most travelers have brief itineraries without exposure to at-risk areas, and others may have been immunized. Among 24 cases reported to the Centers for Disease Control and Prevention (Anonymous, 1996) from 1978 through 1992, 11 occurred in expatriates, 8 of whom were U.S. military personnel or their dependents. Among other cases in Americans, only one was in a tourist, one was in a summer student, and in one case the exposure history was unknown. Annual incidence in American travelers can be estimated roughly at well under 1 per 1 million.

Risk can be extrapolated from attack rates in unimmunized American, Australian, and British soldiers exposed in Asia. Rates have

ranged from 0.05 to 2.1 per 10,000 per week in soldiers who were exposed intensely under field conditions, in some instances during epidemics (Sabin, 1947; Sabin and Schlesinger, 1947; Pond and Smadel, 1954; Ognibene, 1982). These high rates could apply to high-risk travelers for a 5-month period (May–September north of the equator and and October–February south of the equator) in most areas of SE Asia.

Travelers can lower risk further by wearing mosquito repellant and long-sleeved shirts and trousers, by avoiding outdoor activities in the evening, and by sleeping under permethrin-impregnated mosquito nets or in screened or air-conditioned rooms (Tsai *et al.*, 1997).

IX. Risk Factors

A. *Host Factors*

By virtue of high levels and lengthy periods of viremia after infection and high population in some areas, pigs are key hosts for viral amplification. Infections in adult pigs are asymptomatic, but during pregnancy infection frequently results in abortions and stillbirths with significant economic losses. In some locations enzootic transmission of the virus is maintained among birds, and in well-characterized outbreaks in which pigs were absent, such birds have served as epidemic amplifying hosts (Phanthumachinda, 1995; Soman *et al.*, 1977). Other domesticated animals, such as cattle, dogs, sheep, cows, and chickens, and peridomestic rodents may become infected, but viremia levels may be insufficient to support amplification (Ilkal *et al.*, 1988). JE mosquito vectors are zoophilic; consequently, cows and certain other animals can reduce risk to humans by diverting biting activity of vector mosquitoes (zooprophylaxis) (Takahashi *et al.*, 1971). Immunization of pigs prevents abortion and stillbirths and also may serve to reduce viral transmission by removing pigs as viral amplifiers (Sasaki *et al.*, 1982; Takahashi *et al.*, 1971; Vaughn and Hoke, 1992). Experimental immunization of nearly the entire pig inventory on one island in Japan led to a significant reduction in human cases (Takahashi *et al.*, 1971). However, pig immunization has shown to be difficult to maintain so it has not been continued as an ongoing control measure (Igarashi, 2002).

The great majority of JE infections are not apparent. In susceptible non-indigenous adults, only one in 25–63 infections resulted in symptomatic illness (Benenson *et al.*, 1975; Halstead and Grosz, 1962). In Asian children, the ratio has been estimated at 1:500 or higher (Southam, 1956). These observations suggest the existence of a JE

resistance gene in indigenous Asians who have been exposed to JEV infections presumably over many millennia. JE is reported more often in males than females, but this could reflect greater outdoor exposure of boys. JE attack rates are inherently higher in children than in adults. Case attack rates in the 1947–1948 Guam outbreak were five times higher in children younger than 16 years of age than in adults (Hammon *et al.*, 1958). In addition, predominance in children is attributable to the fact that in many areas the population has all been exposed, by young adulthood, with nearly all demonstrating serology (Tsai, 1999). Transmission in all age groups indicates the virus is more recently introduced into our area (Sabchareon and Yoksan, 1998).

In countries where cases in children have been prevented by immunization, the age distribution of cases has shifted toward adults, and particularly to the elderly. In Japan the previous bimodal age distribution of cases, with peaks in young children and in the elderly, has shifted toward a predominance of cases in adults (Oya, 1989). A similar pattern holds in Korea and in developed municipalities of China. This, however, does not appear to represent an increase in the actual number of cases in the elderly but with decreased cases in the younger immunized age-groups, cases in the elderly predominate. In Taiwan, age-specific incidence is highest in adults 20 to 39 years old, probably because this cohort is too old to have been immunized fully when mass vaccinations began in 1968 and too young to have acquired infections naturally in a newly industrialized society.

Behavioral and other factors associated with the risk of acquiring JE vary regionally. Household crowding, religion, ethnicity, exposure to domestic animals, and lack of air conditioning were identified as risk factors in some studies (Chaudhuri *et al.*, 1992; Hiroyama, 1962; Paul *et al.*, 1993). Use of permethrin-impregnated mosquito nets, but not untreated nets, is protective (Dapeng *et al.*, 1994). Although risk for acquiring JE is greatest in rural areas, conditions that permit enzootic viral transmission exist within or at the periphery of many Asian cities. For example, JE cases in Taiwan are reported principally from areas surrounding Taipei; in Vietnam, JE incidence is highest in and near Hanoi; and in India, urban outbreaks have occurred in Lucknow. Cases have been reported from residents of Beijing with no history of travel to rural areas (Xu *et al.*, 1996).

The loss of vaccine-derived immunity can be inferred from declining JE antibody prevalence rates, from 49% in primary school to 38% in junior high school, 34% in junior college, and 29% in university students (Wu *et al.*, 1999). It is not clear how many JE cases in adults accompany this antibody decline. There is some evidence gathered during the era

before the introduction of vaccine that encephalitis occurs in some persons who were presumably protected earlier in life by antibodies raised by silent wild-type viral infections. This was evidenced by the observation that some JE cases in the elderly have been accompanied by secondary type antibody responses (Ishii *et al.*, 1968). For most of the vaccine era, Japan, China, Korea, and Taiwan adopted pro-active booster immunization policies with the manufacturers themselves establishing immunization schedules. The protection afforded by administering multiple booster doses has not been studied, but may have contributed to the high level of protection against JE observed in entire populations. At present there is a trend to administer fewer booster immunizations. This change in policy may provide important data on the long-term protective efficacy of a few doses of killed JE vaccine.

B. Viral Factors

Evidence that overt encephalitis is a rare outcome of JEV infection supports a hypothesis that a majority of circulating strains are attenuated for human beings, only a small faction being fully neurovirulent. There is no laboratory evidence that this is the case. Neurovirulence of JE strains recovered from mosquitoes and reservoir hosts for mice is identical to that of strains recovered from human encephalitis cases (Huang, 1957). There are no genetic differences in JEV strains recovered from mosquitoes or reservoir hosts and human autopsy materials in any given area (Chen *et al.*, 1990). The high ratio of inapparent to overt disease, rather, emphasizes the fact that still unknown host factors determine the outcome of individual infections.

When it was observed that isolates from northern and southern Thailand were of different genotypes, it was suggested that genotype differences might correlate with disease endemicity, north and south (Chen *et al.*, 1990). However, data from Vietnam do not support this. Isolates of the virus from epidemic northern Vietnam were the same genotype as those from endemic southern Vietnam (Huong *et al.*, 1993).

X. Ecological Factors in Disease Transmission

Field studies and mathematical models suggest that the magnitude of JE outbreaks is related directly to the density of vector and not the number of viremic amplifier hosts (Wada *et al.*, 1975). In addition, there may well be focal “hot spots” of JEV transmission around piggeries.

In developed Asian countries (e.g., Japan and Korea), JE incidence has decreased over several decades to fewer than five cases annually (Fig. 5). A number of factors other than immunization have contributed to the decline, including improved standard of living resulting in screened houses and air conditioning, a reduction in land under cultivation, and changes in agricultural practices, especially the increased use of pesticides and centralized pig production. The impact of economic development has been demonstrated most clearly in Singapore, which has no national immunization program. Although JE previously was endemic on the island, no indigenous cases have been detected since 1992, and serosurveys have shown no antibodies in children younger than 12 years of age, indicating the near elimination of viral transmission through environmental changes, mosquito control, and complete prohibition of pig rearing on the island (Goh, 1996). Although imported pigs are held briefly in quarantine, their segregation from the human population may have had a major impact on viral amplification. Although this demonstrates successful environmental control, this model is not realistically reproducable for most other sites in Asia.

The difference in transmission between endemic year-round transmission and seasonal peaks appears to play a role in the recognition of JE as a public health problem. In places with a distinct seasonal peak, JE is recognized as a specific illness that then demands public health attention; however, where transmission is year-round the disease may go unnoticed. In Bali, Indonesia, cases of JE were first recognized in tourists returning from vacation. This led to further investigations in Bali where JE has now been demonstrated to be a significant health problem with year-round transmission (Xu, 2001, personal communication).

A. Season

JE is transmitted in epidemics or in an endemic pattern, or both, in virtually every country of Asia. Officially reported cases underestimate the magnitude and geographic extent of risk because of underreporting or, in some countries, widespread immunization. Transmission is seasonal, occurring approximately from June to September in temperate areas of China, Korea, Japan, and far eastern Russia. Farther south, the transmission season is somewhat longer, extending from March through October. In tropical areas of Southeast Asia and India, seasonal transmission is particular to local patterns of monsoon rains and bird migration, with the possibility of two transmission intervals

in a calendar year. The virus is transmitted throughout the year in some sites. However, viral persistence in vertebrate hosts, such as bats and reptiles, and annual reintroductions of the virus through migrations of birds or windborne mosquitoes also has been hypothesized as a mechanism by which endemic foci are maintained (Ritchie and Rochester, 2001). Self-limited outbreaks on Western Pacific islands, on Guam in 1947, Saipan in 1990, and the Australian Torres Strait islands in 1995 and 1998 were examples of viral introductions possibly by migratory birds or, in the last case, by windblown mosquitoes (Hanna *et al.*, 1996, 1999; Paul *et al.*, 1993; Ritchie and Rochester, 2001).

In temperate regions vector mosquitoes emerge in May, and after several initial rounds of viral amplification, high rates of pig seroconversion are detected, followed almost immediately by the onset of human cases, typically in July and August. In rural villages all elements of the enzootic transmission cycle are found in proximity to human residences and activities (Fig. 12). Consequently, exposure and infection occur at an early age. In areas where transmission is hyperendemic, half of all cases occur in children younger than 4 years of age, and nearly all cases are found in children younger than 10 years.

FIG 12. Animal husbandry in Bali, Indonesia, showing proximity of pig pen to human habitation. (See Color Insert.)

B. Transmission Cycles

As established in a series of studies by Buescher and colleagues and Gresser and colleagues (Buescher *et al.*, 1959a; Gresser *et al.*, 1962), *Culex tritaeniorhynchus summarosus* is generally regarded as the principal vector of zoonotic and human JE transmission in northern Asia. This mosquito is a nighttime biting mosquito that feeds preferentially on large domestic animals and birds but only infrequently on humans (Fig. 13). In Japan, Okinawa, Korea, and China, JE virus has been recovered infrequently from other culicine mosquitoes, including *Culex pipiens* (Buescher *et al.*, 1959a; Hurlbut and Nibley, 1964; Matsuyama *et al.*, 1960; Shichijo *et al.*, 1968). Epidemiologic evidence has been adduced to suggest a role for *Aedes togoi* in the transmission of JE virus in the Far Eastern maritime provinces of Russia (Rosen, 1986). Isolation from *Aedes vexans nipponii* has been reported from Japan (Shichijo *et al.*, 1968).

A more complex ecology prevails in southern Asia. From Taiwan to India, JE virus has been isolated frequently from *Culex tritaeniorhynchus* in most areas of Asia, but various species, principally ground pool– and rice paddy–breeding, including *Culex vishnui*, *Culex pseudovishnuri*, *Culex gelidus*, *Culex fuscocephala*, *Culex bitaeniorhynchus*, *Culex infula*, *Culex whitmorei*, and *Culex annulus*, are also important locally. JE virus has been recovered from *C. pipiens pallens* and

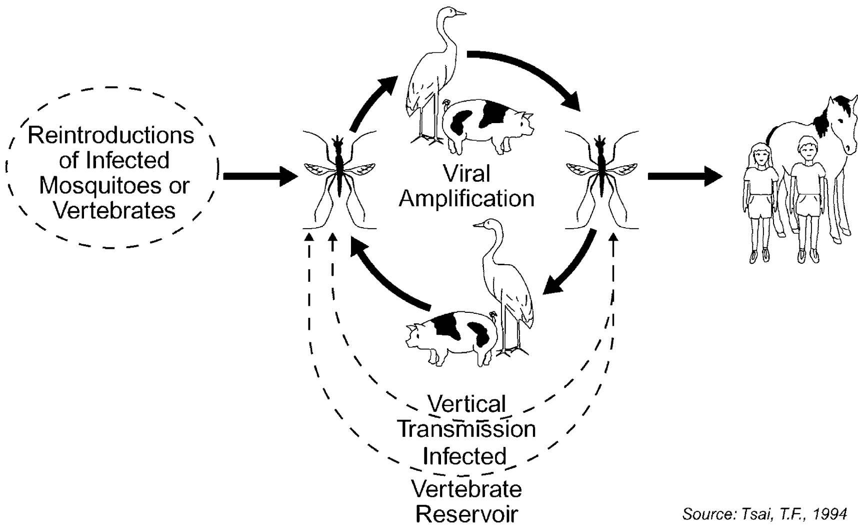


FIG 13. Cycle of Japanese encephalitis in nature (Tsai *et al.*, 1999).

C. quinquefasciatus in urban locations. In addition, *Culex annulirostris* has been identified as a vector in the Western Pacific, *Aedes togoi* in sylvatic locations in Siberia, and members of the *Anopheles hyrcanus* group in northeastern India (Ritchie *et al.*, 1997; Sunish and Reuben, 2001). In Indonesia JE has been isolated from at least 11 different species of mosquitoes, including *Anopheles annularis*, *Anopheles kochi*, *Anopheles vagus*, and *Armigeres subalbatus*.

In northern Asia, *C. tritaeniorhynchus* larvae are found in rice fields, marshes, and small, stable collections of clean water around cultivated fields. This warm weather mosquito is most abundant in July, August, and September in subtropical and temperate climates. The adult females do not rest in houses or animal shelters in the daytime except in small numbers. Entry and resting at night in houses and animal shelters have been observed, but feeding probably takes place most often in the open. Feeding activity is greatest during the first hour after sunset. In temperate areas, cold weather interrupts transmission.

JE is principally a disease of rural areas in which vector mosquitoes proliferate in close association with pigs, wading birds, and ducks, the principal vertebrate amplifying hosts (Burke and Leake, 1988; Igarashi, 1992; Rosen, 1986; Scherer and Buescher, 1959) (Fig. 13). Humans and horses may become ill after infection, but such illness is incidental to the transmission cycle (Gould *et al.*, 1964; Wang *et al.*, 1982). Experimental observations and field studies indicate that the virus can overwinter in vertically infected mosquitoes (Min and Xue, 1996). Although vector abundance and risk for human infection are associated with rainfall, with increased implementation of irrigation in rice cultivation, paddy flooding schedules increasingly influence vector bionomics. Single paddies can produce more than 30,000 adult mosquitoes in a day. Collectively, these artificial breeding sites overpower the impact of other natural breeding sites. Because of this, mosquito abundance fluctuates with periodic rice field flooding and can peak at any time of the year, including the dry season (Olson *et al.*, 1983, 1985; Ritchie and Rochester, 2001) (Fig. 14).

C. Maintenance and Overwintering

An important question asked for many years is how JE virus survives the winter months in temperate countries. Reported data supports any or each of four options. The virus may overwinter in hibernating mosquitoes, in mosquito eggs via vertical transmission, in bats and various cold-blooded animals that hibernate during cold weather, or it may be introduced from areas of year-round transmission by migrating birds.

FIG 14. Rice paddies, Bali, Indonesia. (See Color Insert.)

C. tritaeniorhynchus survives the winter in the adult form, hibernating in protected areas. Experimental and field studies demonstrate that JE virus survives winters in some hibernating adults (Hayashi *et al.*, 1975; Mifune, 1965; Omori *et al.*, 1965) (Fig. 15). In Korea, a collection of 50,499 mosquitoes over a 6-year period yielded two strains of JE virus (Lee, 1971). Virus also appears to pass vertical transmission from adult to eggs that survive the winter (Rosen *et al.*, 1980, 1989). If infected adults or infected freshly hatched mosquitoes emerge with the coming of spring, an unanswered question raised by these observations is why detectable transmission is delayed until mid-summer.

Although much interest has centered on surviving infected adult mosquitoes as an overwintering mechanism for JE virus, this is not a proven major mechanism of virus survival. Several poikilothermic hibernating vertebrates have been found to support JE virus infection experimentally. These include several species of bat (Sulkin *et al.*, 1963; 1964), gekkos (Toda *et al.*, 1972) and other lizards (Doi *et al.*, 1983), and frogs. Snakes and turtles also may become infected and circulate HI and neutralizing antibody (Shortridge *et al.*, 1974; Shortridge *et al.*, 1977). As a maintenance reservoir of JE virus, the strongest case has been made for bats. Circulating virus was found in the blood of bats that were rewarmed following hibernation at low

FIG 15. Monitoring Japanese encephalitis vector mosquito populations, Andhra Pradesh, India. (See Color Insert.)

temperatures. Further, JE virus passes the placenta and can establish infections in infant bats (Sulkin *et al.*, 1964). JE virus was recovered from the organs of wild Japanese bats at all seasons of the year (Sulkin *et al.*, 1970).

XI. FUTURE DIRECTIONS

JE is perhaps the most severe human pathogen of the widespread arboviral zoonoses. Much remains to be learned about its pathogenetic mechanisms, how to improve treatment and rehabilitate encephalitis cases. An inventory of effective antivirals could have a place in preventing severe and disabling disease. The mainstay for coping with JE remains prevention through vaccination.

As evidenced by its ability to replicate in a wide range of vertebrate and invertebrate species, JEV is one of the most protean of animal viruses, rivaling West Nile virus in this regard. The molecular basis for this broad-based cellular tropism requires study as does its complex zoonotic behavior. Close attention will need to be given to the expansion of JE in our global world as much remains to be learned about the interaction of zoonoses and our rapidly changing environment.

As part of the process of justifying the expense of national immunization programs, data should be collected on the continuing risk in endemic and new transmission areas. As surveillance lags and disease risk and recognition decrease, immunization programs can fail as has been seen with yellow fever in Africa. There is a danger that the disappearance of clinical disease may reorder priorities, leading governments to reduce support for JE vaccine programs. They may also discontinue JE research. This may prove to be a costly mistake. JE is a zoonosis. Eradication is theoretically not possible. In truth, each year 3 billion human beings are at risk for JE and should be fully immunized. One billion of these persons are reached by current vaccination programs but much work remains to be done to reach the remaining 2 billion people who live at risk. Vaccines capable of protecting humans from JE have existed since 1941. It is now time to assure that effective and safe vaccines are given to all persons at risk for this devastating disease (Endy and Nisalak, 2002).

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ADVANCES IN VIRUS RESEARCH, VOL 61

EPIDEMIOLOGY OF ST. LOUIS ENCEPHALITIS VIRUS

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An excellent book (Monath, 1980b), a detailed regional monograph (Reeves, 1990a), and several informative recent reviews (Monath and Tsai, 1987; Tsai and Mitchell, 1989; Day, 2001) have provided thorough overviews of the epidemiology and ecology of St. Louis encephalitis (SLE) virus. The current chapter extends these earlier presentations to emphasize epidemiologic events and research during the past decade that targets important unsolved aspects of SLE epidemiology, historically one of the North America's most important arboviruses.

I. History

With the exception of West Nile virus, the North American encephalitidies, including SLE, probably have been present in the New World and transmitted within their bird-mosquito cycles for thousands of

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years. For much of this period human populations were sparse, limiting the number of infections and the impact of this disease on native Americans. The arrival of European settlers in the 1600s and the extensive agricultural development that followed greatly altered the landscape by clearing vast areas of North America and probably increased the abundance of *Culex* mosquito vectors, avian hosts such as house finches and mourning doves, human-mosquito contact, and the prevalence of infection. However, because of the varied clinical picture and the absence of specific laboratory diagnostic methods, disease caused specifically by arbovirus infections such as SLE most assuredly was confused with other agents causing central nervous system (CNS) disease during summer.

During 1933, a major epidemic with more than 1000 cases of encephalitis occurred in St. Louis, Missouri (Lumsden, 1958). These cases occurred during the middle of an exceptionally hot, dry summer and were concentrated within areas of the city adjacent to open storm water and sewage channels that produced an extraordinarily high abundance of *Culex* mosquitoes. A virus, later named St. Louis encephalitis (SLE) virus, was isolated from suspensions of brain specimens from deceased patients by intracerebral inoculation of rhesus monkeys (Muckenfuss *et al*., 1934) and then white mice (Webster and Fite, 1933). Mouse protection by convalescent human sera demonstrated that SLE differed from other viruses causing CNS disease (Webster *et al*., 1933), including equine encephalomyelitis, poliomyelitis, and vesicular stomatitis (Cox and Fite, 1934; Hammon and Izumi, 1942a). The epidemiologic features of this epidemic, including concordance among SLE cases, warm summer temperatures and elevated *Culex* mosquito abundance, remain the hallmark of SLE epidemics to this day.

A unique multidisciplinary investigation of an SLE epidemic in the Yakima Valley of Washington during 1941 and 1942 (Hammon *et al*., 1945a) established the involvement of peridomestic wild birds as reservoir hosts and *Culex* mosquitoes as vectors of SLE (Hammon *et al*., 1941, 1942b). These seminal field studies later were confirmed by parallel investigations in Kern County, California (Hammon *et al*., 1945b) and extended the focus of mosquito surveillance and control in North America from pest *Ochlerotatus* and *Anopheles* vectors of malaria to *Culex* vectors of encephalitis viruses.

Epidemics of encephalitis in the western United States often included a mixture of cases of various etiologies that could not be differentiated clinically (Hammon, 1941, 1943). To definitively separate infections due to SLE, western and eastern equine encephalomyelitis, and poliomyelitis viruses, neutralization assays in mice were

standardized to test acute and convalescent phase sera (Hammon *et al.*, 1942a).

Understanding the basic transmission cycle, an appreciation of the clinical symptoms, and the development of laboratory diagnostic procedures provided an expanding view of the public health importance of SLE, with epidemics or clusters of cases recognized annually throughout the United States. The wide geographical range and intermittent appearance of human cases have been documented thoroughly since 1933, and total more than 1000 deaths, more than 10,000 cases of severe illness, and more than 1,000,000 mild or subclinical infections (Monath, 1980a). The largest human epidemics involving hundreds of cases occurred in Missouri (1933, 1937), Texas (1954, 1956, 1964, 1966), Mississippi (1975), Ohio (1975), Indiana (1975), Illinois (1975), and Florida (1977, 1990). Smaller outbreaks have been recognized in California (1952), Ontario (1975), New Jersey (1962), and several other states. Most outbreaks were distributed south of the 70°F June isotherm (Hess *et al.*, 1963) and were associated statistically with above-normal temperatures in spring and below normal rainfall during summer (Monath, 1980a). Warm temperature rapidly drives SLE through the mosquito population (Reisen *et al.*, 1993b), whereas below-normal rainfall allows the pooling of water within municipal drainage systems and results in large populations of *Cx. pipiens* complex mosquitoes.

The last major SLE epidemic in the United States with greater than 100 clinical cases occurred in Florida during the summer of 1990 (Day, 2001; Meehan *et al.*, 2000). Extensive early season seroconversions in sentinel chickens provided an early warning of pending human infection and triggered an intervention response by mosquito control agencies (Day, 2001). Surveillance activities carefully described the wide distribution of human cases in relation to sentinel chicken seroconversions (Day and Stark, 1996). Ongoing longitudinal studies documented elevated seroprevalence rates in mourning doves (Day and Stark, 1999), and *Cx. nigripalpus* abundance (Day and Curtis, 1993), feeding behavior (Day *et al.*, 1990), and virus infection rates (Shroyer, 1991) during the epidemic period. A total of 222 laboratory-confirmed human cases with 11 deaths were recorded, with an overall attack rate of 2.25 per 100,000 (Meehan *et al.*, 2000). Serosurveys indicated that as many as 3.6% of the central Florida population may have been infected and that infection rates were highest in persons with outdoor occupations (7.1%) and attending homeless shelters (13.3%). Health warnings, the closing of recreational parks such as Walt Disney World at night, and use of personal protection seemed to reduce infection rates (Meehan *et al.*, 2000).

II. Clinical Description and Diagnosis

A. Humans

Like most arboviruses that cause CNS disease, infection with SLE does not result in a clear clinical picture and most infections remain unrecognized, unless associated with an epidemic. When presented with such diverse symptoms, few physicians initially suspect SLE, even in endemic areas. For example, in the summer of 1989 in Kern County, California, there was almost a 100% increase in hospital admissions with encephalitis. Concurrently a countywide medical alert was issued for SLE, because high seroconversion rates were detected in sentinel chickens and high minimum infection rates in mosquitoes (Reisen *et al.*, 1992b). However, not a single human case of SLE was diagnosed and reported until follow-up tests on blood samples from 77 discharged CNS disease patients revealed that 29 had elevated IgM titers indicative of recent SLE infection (Tueller, 1990).

Most SLE infections, especially in young or middle-age groups, fail to produce clinical disease, and infected individuals rarely experience more than a mild malaise of short duration with spontaneous recovery (Tsai *et al.*, 1989). Depending on SLE strain virulence and the infection history of the population, apparent to inapparent infection ratios have been estimated to range from 1:16 to 1:425 following epidemics (Monath, 1980a). Without a history of acquired immunity by the resident population, clinical illness is most severe and most frequent among the elderly. However, in endemic areas with high rates of acquired immunity, illness peaked in children and was low in older residents (Reeves and Hammon, 1962). Overall case-fatality rates range from approximately 5–20% (Tsai *et al.*, 1989), but always are higher among the elderly (>75 years of age), who often present with additional medical complications.

Clinical disease due to SLE infection may be divided into three syndromes in order of decreasing severity: 1) encephalitis (including meningoencephalitis and encephalomyelitis) with high fever, altered consciousness and/or neurologic dysfunction; 2) aseptic meningitis with high fever and stiff neck; and 3) febrile headache with fever, headache possibly associated with nausea or vomiting, and no CNS illness (Brinker and Monath, 1980). The onset of illness may be sudden (<4 days) and acute, leading rapidly to encephalitis, or insidious progressing gradually through all three syndromes (Brinker *et al.*, 1980; Tsai *et al.*, 1989). Symptoms may resolve spontaneously during any stage of the illness with full recovery. Acute illness may be followed by

"convalescent fatigue syndrome" in <50% of patients (Palmer and Finley, 1956), with complaints of general weakness, depression, and the inability to concentrate that generally resolve within 3 years (Bredeck *et al.*, 1938; Palmer *et al.*, 1956). Other sequellae include headache, disturbances in gait, and memory loss (Brinker *et al.*, 1980).

Definitive diagnosis in humans depends almost entirely upon serology. Clinical laboratory tests on cerebrospinal fluid (CSF) show nonspecific findings characteristic of many CNS infections, including increases in polymorphonuclear cells, total cell counts <200/cc, and mildly elevated protein levels (Tsai *et al.*, 1989). Pathologic findings at autopsy are not definitive, although virus and viral antigen may be demonstrated in brain tissue by several standard methods (Gardner and Reyes, 1980) as well as by reverse transcription–polymerase chain reaction (RT-PCR) (Howe *et al.*, 1992; Lanciotti *et al.*, 2000). Presumptive diagnosis by acute CSF or serum specimens is based most frequently on the demonstration of SLE specific IgM antibody using an enzyme immunoassay (EIA). This method was shown to be 71% sensitive in diagnosing cases using sera drawn 0–2 days after the onset of clinical illness (Monath *et al.*, 1984). Indirect fluorescent antibody tests also have been used, but results often are confounded by individual interpretation. IgG antibody against SLE virus can be demonstrated by EIA, hemagglutination inhibition (HI) test, and neutralization tests on Vero cell cultures (plaque reduction neutralization test [PRNT]) or in white mice; titers usually increase within 1 week after clinical onset. Complement-fixing (CF) antibody takes longer to increase in titer (often 4–6 weeks), and therefore may not be useful in clinical diagnosis. Definitive diagnosis is based on the demonstration of a greater than fourfold increase in PRNT or HI titer between acute and convalescent sera. Previous infection with or immunization against other flaviviruses (including Japanese encephalitis or yellow fever) may confound definitive diagnosis due to cross-reactivity and persistence, but this usually can be resolved by comparative end point titration using PRNTs.

B. Vertebrate Animals

Although frequently antibody positive during serosurveys, SLE infection by mosquito bite or subcutaneous (s.c.) inoculation does not produce elevated viremias or cause clinical illness in domestic animals, including equines, porcines, bovines, or felines (McLean and Bowen, 1980). In a single experiment (Furumoto, 1969), dogs (purebred beagles) produced a low-level viremia, with two of eight dogs developing clinical illness. Fowl younger than 1 month old (including chickens

and ducks) consistently developed sufficient viremia to infect mosquitoes (Hardy and Reeves, 1990a; McLean *et al.*, 1980, 1985a), but, similar to adults, did not develop clinical illness. Adult chickens (>22 weeks old) usually failed to develop a detectable viremia, and along with immature birds developed long-lasting EIA, neutralizing and HI antibodies (McLean *et al.*, 1980; Reisen *et al.*, 1994).

Similar to domestic fowl, wild birds did not develop apparent illness following infection by s.c. inoculation or mosquito bite (McLean *et al.*, 1980). The viremia response varied markedly, depending on virus strain (Bowen *et al.*, 1980), bird species (McLean *et al.*, 1980), and bird age (Bowen *et al.*, 1980), but typically was short-lived and detected from days 1 to 5 post-infection. The geographical origin and isolation history of virus strains was critical to their producing a viremia response in adult house sparrows (Bowen *et al.*, 1980). Strains isolated from *Cx. pipiens* complex mosquitoes from the central and eastern United States produced elevated viremias, whereas strains isolated from *Cx. tarsalis* from the western United States were weakly viremogenic. Similar to domestic fowl, immature birds produced a higher viremia in response to infection than adult birds. Regardless of viremia response, most birds produced antibody detectable by HI, EIA, or PRNT. In house sparrows, a single infection was protective against re-infection for up to 2 years (McLean *et al.*, 1983), whereas in one experiment, three of six house finches rechallenged with a heterologous virus strain 1 year after infection produced fleeting viremias that probably were sufficient to infect susceptible mosquitoes (Reisen *et al.*, 2001b). Birds also produced an IgM response to infection (Calisher *et al.*, 1986); however, in some species such as house sparrows, these antibodies disappeared within 3 weeks, limiting their use in surveillance (McLean *et al.*, 1985a).

The responses of mammals to natural or experimental infection have varied. Serosurveys occasionally have shown higher SLE prevalence rates in mammals than birds (McLean *et al.*, 1980), but this could be confounded by comparative survival rates. White mice (*Mus musculus*) of different ages respond differentially to intracerebral (i.c.) and intraperitoneal (i.p.) inoculation of SLE virus, and this response has been used as a model to measure virulence and neuroinvasiveness (Monath *et al.*, 1980a). Rodents in the genera *Ammosperophilus* and *Dipodomys* were susceptible to infection after s.c. inoculation, whereas *Spermophilus*, *Rattus, Sigmodon*, and *Peromyscus* were refractory (Hardy and Reeves, 1990b; McLean *et al.*, 1985a). Similarly varied were lagomorphs; *Lepus* was susceptible (Hardy *et al.*, 1990b), whereas four species within *Sylvilagus* ranged from refractory to susceptible (Hardy

et al., 1990b; McLean *et al.*, 1985a). Raccoons and skunks were refractory, whereas opossums and woodchucks were susceptible (McLean *et al.*, 1985a). Like birds, susceptible mammals produced an immediate viremic response that generally persisted for less than 1 week, and all species produced detectable antibodies regardless of their viremia response. SLE frequently has been isolated from bats (*Tadarida*, *Myotis*, etc.), and many populations exhibit a high prevalence of neutralizing antibody (Allen *et al.*, 1970; McLean *et al.*, 1980). Bats entering hibernation produced lasting infections that relapsed after hibernation was terminated naturally or artificially (Sulkin and Allen, 1974) and therefore could be important in SLE overwintering. Overall, the role of mammalian infection in SLE epidemiology is complex and difficult to interpret. All reputed *Culex* vectors feed most frequently on avian hosts, occasionally on large mammals and lagomorphs, rarely on rodents, and almost never on bats (Edman, 1974; Reisen and Reeves, 1990; Tempelis, 1975). In addition, rodents and bats are infected with a large number of other *Flaviviruses*, possibly confounding serologic testing (Varelas-Wesley and Calisher, 1982).

Diagnosis of animal infection by virus isolation is infrequent, and serology may be complicated by host-species diversity. Peripheral viremias in most adult mammals and birds are transient (1–5 days) and at fairly low titer (<10,000 plaque-forming units [PFU] per milliliter of blood), making isolation difficult from field populations. Viremias in nestling birds typically are higher and isolations have been made most frequently from immature birds (McLean *et al.*, 1980). In comparative studies of blood and other tissues from experimentally infected white-crowned sparrows, virus was isolated readily from all organs by plaque assay on Vero cells and was detected with RT-PCR assay when birds exhibited a detectable viremia (Kramer *et al.*, 2002). RT-PCR was more sensitive than plaque assay, although infectious virus was detected readily by plaque assay on Vero cells after passage in C7/10 and/or C6/36 *Aedes albopictus* cells. Serologic assays generally have relied on HI or PRNT, because there is no requirement for host-specific reagents. EIAs specific for IgM seem cross-reactive among avian species (Calisher *et al.*, 1986), but in some species IgM seems to be present for a short period thereby limiting probability of detection. Reagents produced for chicken-specific EIAs have been useful in surveillance programs (Olson *et al.*, 1991), and reputedly are cross-reactive with additional bird species (Tsai and Mitchell, 1989). This EIA technology has been simplified for surveillance by the transfer of antigen to polyvinylidene difluoride strips (Oprandy *et al.*, 1988). Recently, a sandwich EIA using a broadly reactive anti-bird detector antibody

conjugated to a horseradish peroxidase system has proven useful in detecting antibodies in almost all bird species to any pathogen (including SLE) (Chiles and Reisen, 1998).

III. Geographical Distribution and Genetic Variation

SLE virus is distributed from southern Canada south through Argentina and from the west to the east coasts of North America and into the Caribbean Islands (Monath, 1980a; Spence, 1980). Historically, human cases have been detected in Ontario and Manitoba, Canada, all of the continental United States (except the New England States and South Carolina), Mexico, Panama, Brazil, Argentina, and Trinidad. The low number of human cases in Canada reflects the warm temperature requirements for SLE virus growth in the mosquito host, whereas the low numbers of cases from tropical America may reflect inadequate laboratory diagnosis, the circulation of attenuated virus strains, and/or enzootic cycles involving mosquitoes that feed infrequently on humans (Spence, 1980). Additional information to support this distribution comes from SLE isolations from birds, mammals, and mosquitoes as well as serosurveys of mammal and avian populations.

SLE virus consists of a positive-sense, single-stranded RNA enclosed within a capsid composed of a single polypeptide (C) and surrounded by an envelope containing one glycosylated (E) and one nonglycosylated (M) protein (Calisher, 1994). The E protein is most variable and associated with hemagglutinating and neutralizing activities. Detailed studies during the mid-1980s clearly demonstrated geographic variation among SLE strains using oligonucleotide fingerprinting (Trent *et al.*, 1980) and virulence markers (Bowen *et al.*, 1980; Monath *et al.*, 1980a). The 43 strains investigated were grouped into six clusters: 1) east central and Atlantic United States, 2) Florida epidemic, 3) Florida enzootic, 4) eastern United States, 5) Central and South America with mixed virulence, and 6) South America with low virulence. These clusters also were supported by the presence or absence of human cases.

Recent developments in molecular genetics have extended our understanding of SLE genetics and distribution and have provided important insight into virus ecology. Sequences of the envelope gene from SLE strains isolated from California from 1952 through 1995 varied temporally and spatially (Kramer *et al.*, 1997), indicating regional persistence in the Central Valley for at least 25 years as well as sporadic extinction and introduction. Similar studies in Texas (Chandler *et al.*,

2001) using a single-strand conformation polymorphism (SSCP) technique showed that multiple SLE strains may circulate concurrently and remain highly focal, whereas other strains amplify and disseminate aggressively during some summers, but then disappear. Phylogenetic analyses of sequence data from 62 SLE isolates made throughout the known geographical range of SLE (Kramer and Chandler, 2001) indicated that there are seven lineages that overlap somewhat with the six groups defined above by oligonucleotide fingerprinting: 1) western United States, 2) central and eastern United States and three isolates from Mexico and Central America, 3) one mosquito isolate from Argentina, 4) five isolates from Panama mosquitoes, 5) South American strains plus an isolate from Trinidad, 6) one Panama isolate from a chicken, and 7) two isolates from Argentina rodents. Collectively, these data indicated that some SLE strains remained almost unchanged within regional foci, some for periods >25 years (Kramer *et al.*, 1997), whereas others have been transported between areas within and outside the United States. However, the mechanisms for both persistence and long distance transport have not been resolved. The current genetic data cannot delineate mechanisms that would lead to local maintenance and the emergence of new strains or to local extinction and annual re-introduction of similar genotypes from the same or similar localities.

IV. Incidence and Prevalence

During the 25-year period from 1976 to 2000, 1434 human cases were reported to the Centers for Disease Control and Prevention (mean, 57.4 per year; coefficient of variability (C.V.), 140.2). Most clinical cases have been reported from Texas, Florida, the southeast, and the Ohio River drainage (Fig. 1). When these cases were averaged and incidence estimated using population data from the 1990 census, the highest attack rates seemed to be focused in the southern states from Texas through Florida bordering the Gulf of Mexico. This geographical pattern of incidence was somewhat different from that estimated for the 1964–1984 period (Tsai and Mitchell, 1989) and that indicated by the distribution of early epidemics (Hess *et al.*, 1963). Cases were clustered temporally in the Mississippi and Ohio River drainages between 1976 and 1980, Texas in 1976 and 1980, and Florida in 1977 and 1990 (Fig. 2). Texas had 29% of the total U.S. cases and the most consistent endemic transmission of SLE virus to humans, with 1 to 121 cases reported annually during 21 of the past 25 years (mean, 18 per year; CV, 53.4). In contrast, 333 of the 377 cases reported

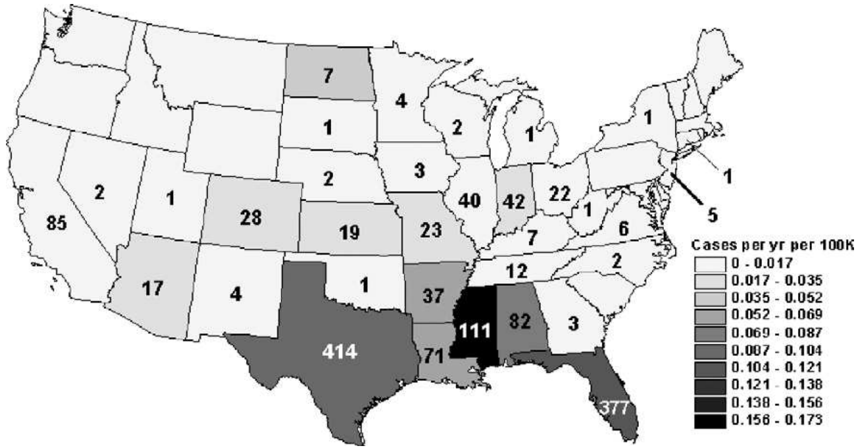


Fig 1. Number of human SLE cases reported to the CDC between 1976 and 2000 and incidence per year per 100,000 population calculated from the 1990 census. (Data from Campbell, G. L., Centers for Disease Control and Prevention, Fort Collins, Colorado, personal communication, 2001).

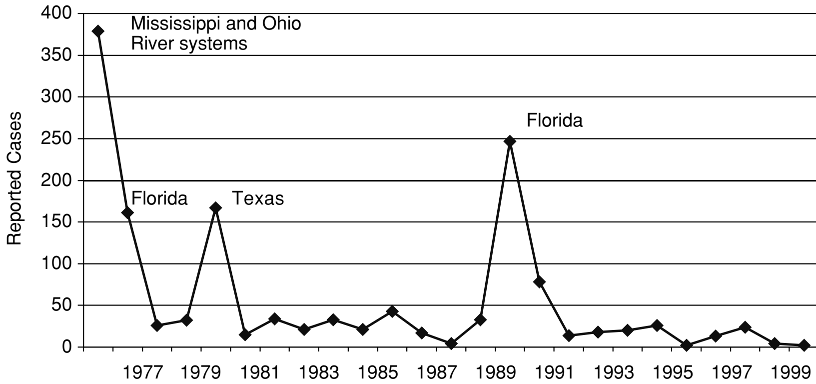


Fig 2. Number of human SLE cases reported per year to the CDC between 1976 and 2000. (Data from Campbell, G. L., Centers for Disease Control and Prevention, Fort Collins, Colorado, personal communication, 2001).

from Florida were reported during two epidemic years. Similarly, cases in California were clustered around outbreaks in Los Angeles in 1984 (Murray *et al.*, 1985) and in Bakersfield in 1989 (Tueller, 1990). Following the 1989 outbreak centered in Bakersfield, SLE activity was detected in 1990 and then in 1992, but then has not been detected

north of the Tehachapi Mountains, although it has been detected enzootically during most years in SE California (Reisen *et al.*, 2002b). Although human cases have not been recognized frequently, serosurveys in Imperial Valley showed that 11% of the population had PRNT antibody against SLE and that by age 70 approximately one of three residents had been infected (Reisen and Chiles, 1997). In contrast, seroprevalence rates were <1% in residents of the Sacramento Valley (Reisen *et al.*, 1997) and 1.7% in residents of Los Angeles following the 1984 outbreak (Kerndt *et al.*, 1988). These seroprevalence estimates differed markedly from historical estimates for SLE in the southern San Joaquin Valley during 1943–1952, when by age 20 approximately 25% of adults had been infected (Reeves and Hammon, 1962) and indicated that 1) disease in the west was no longer widely endemic, and 2) in the event of an epidemic, few individuals would be protected by previously acquired immunity.

V. Risk Factors

Factors associated with the risk of clinical illness include length and place of residence, age, occupation, socioeconomic status, season, and weather.

A. Residence

Clearly, place of residence markedly affects risk of infection, with geographic regions in the southern United States having the greatest numbers of human cases and greatest incidence of disease (Fig. 1). Virus strains from this geographical area also exhibited greater neurovirulence than strains from the western United States or South America based on experimental infection patterns in laboratory mice (Monath *et al.*, 1980b). Because of mosquito abundance relative to humans and host selection patterns, urban residents seem to be at greater risk for SLE infection than are rural residents. However, these conclusions may be confounded by protective immunity acquired early in life that may be greater among rural residents (Reeves *et al.*, 1962) and by low apparent: inapparent case ratios that require a substantially large population to produce clusters of human cases. Seroprevalence rates in endemic areas increased as a function of years of residence (Froeschle and Reeves, 1965; Reisen *et al.*, 1996, 1997), perhaps limiting apparent infection rates to immigrants. Interestingly, new, predominantly Hispanic immigrants to the Imperial Valley of

California had lower antibody rates than long-time residents, indicating that most immigrants probably were being infected locally (Reisen *et al.*, 1997).

B. Age

In the absence of acquired immunity, clinical illness and fatality rates, but not necessarily infection rates with SLE virus, increase dramatically with age. Infection seems to occur equally among different age classes as indicated by the increase in antibody as a function of age in endemic areas (Froeschle *et al.*, 1965) and by cohort seroconversion rates determined after epidemics in previously unexposed populations (Monath, 1980a). For example, using data from the 1964 Houston, Texas, epidemic, seroprevalence rates remained similar among cohorts, whereas the case incidence rates increased from 8.2 per 100,000 for the 0- to 9-year-old cohort to 13.5–27.6 for the 10- to 59-year-old cohorts and to 78.0 for the >60 year old group; apparent-to-inapparent ratios decreased concomitantly from 1:806 to 1:490–1:239 and to 1:85, respectively (Monath, 1980a). Case-fatality rates among 2288 cases reported to the CDC from 1971 to 1983 increased from <6.7% for 0- to 64-year-old age classes, to 9.5% for the 65- to 74-year-old age class, to 18% for the >75-year-old age class (Tsai *et al.*, 1989). The declining prognosis among the elderly probably relates both to increasing severity of symptoms (Brinker *et al.*, 1979) and to complications related to other health problems. The geographical distribution of the elderly U.S. population (Fig. 3) delineates the distribution of the U.S. population potentially at greatest risk of clinical disease, but with the exception of Florida, does not overlap well with the numbers of cases or the incidence of disease (Fig. 1), indicating that other factors must correlate strongly with disease occurrence.

C. Occupation

In the West, where historically SLE was considered a rural disease, infection was related to occupation, with greatest risk among male agricultural workers who frequently lived in suboptimum housing and worked at night (Jozan, 1977; Reeves *et al.*, 1962). However, examination of infection patterns during recent outbreaks (Murray *et al.*, 1985; Tueller, 1990) and serosurveys (Reisen and Chiles, 1997) have failed to show this occupation-related bias in infection. In fact, case incidence during recent urban epidemics indicated that attack rates were highest among elderly women (Monath *et al.*, 1987). However,

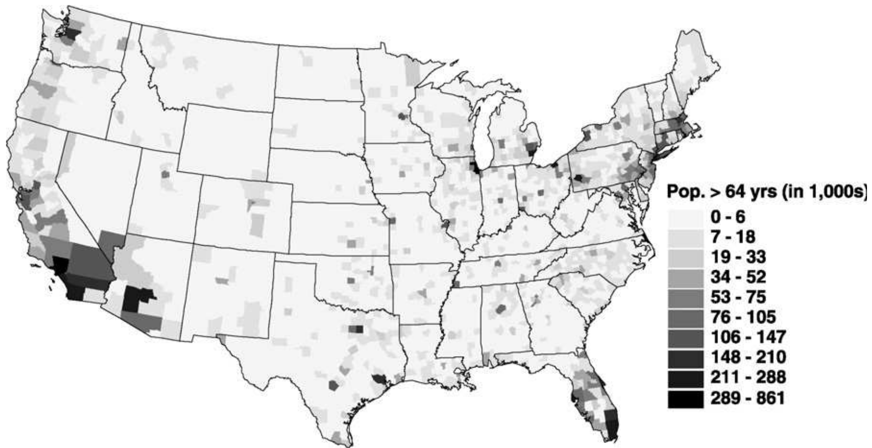


FIG 3. Geographical distribution of Americans older than 64 years of age (data from 1990 census).

outdoor occupation again was identified as a risk factor in the 1990 Florida epidemic (Meehan *et al.*, 2000). These data indicated that there may be differences in risk related to vector species, with elderly women infected most readily during urban outbreaks associated with the *Cx. pipiens* complex and with people working outdoors at greatest risk during rural outbreaks associated with *Cx. tarsalis* or *Cx. nigripalpus*.

D. Socioeconomic Status

Historically socioeconomic status has been related closely to the distribution of cases during epidemics. Homes and municipal drainage systems frequently are not well maintained in low-income neighborhoods, and this may relate to the distribution of human cases, but not necessarily the occurrence of virus within the enzootic transmission cycle. During the 1933 epidemic in St. Louis, for example, cases were clustered among residents living in lower income housing near inadequate open drainage/sewage ditches that produced large *Culex* populations (Lumsden, 1958). Conversely, during surveys of residential neighborhoods in the greater Los Angeles area during 1987–1988, *Cx. p. quinquefasciatus* and *Cx. stigmatosoma* were more abundant in extensively landscaped and irrigated upper socioeconomic level neighborhoods than in lower income neighborhoods with minimal landscaping (Reisen *et al.*, 1988). In 1964 in Houston, Texas, SLE

antibody prevalence was related inversely to the extent of home screening and air conditioning (Luby *et al.*, 1967); a finding confirmed during the 1986 epidemic (Tsai *et al.*, 1988). Similarly, in Bakersfield, California, the overall decline in SLE cases over time paralleled the concurrent increase in air conditioning and television ownership (Gahlinger *et al.*, 1986); residents had changed their evening behavior from sitting on the porch and other outside activities to viewing television within air conditioned and screened housing, thereby reducing vector contact and the risk of infection.

E. Season

The intensity of enzootic transmission and therefore the risk of human infection are affected markedly by temperature fluctuation, manifested by both season and weather. Human cases of SLE almost always occur from mid to late summer into early fall (Monath, 1980a; Reeves *et al.*, 1962; Tsai *et al.*, 1989). Frequently, detection of early season enzootic amplification is a harbinger of epidemics later in the season (Reeves *et al.*, 1962). Because most time during the SLE transmission cycle is spent within the mosquito host, the efficiency and rate of virus amplification and tangential transmission to humans depends heavily on mosquito body temperature that roughly parallels ambient conditions (Meyer *et al.*, 1990). The duration of the extrinsic incubation period decreases and the rate of transmission increases as a function of temperature (Hurlbut, 1973; Reisen *et al.*, 1993b), and these relationships delineate the effective length of the virus transmission season (Reisen *et al.*, 1993b). Human cases tend to occur relatively late within this transmission season (Monath, 1980a) after considerable virus amplification within the primary cycle, but then subside with the onset of cool weather that slows virus replication and initiates vector diapause in response to shortening day length (Bellamy and Reeves, 1963). Extraordinarily cold weather also may impact virus occurrence. A widespread freeze in central Florida during the winter of 1989–1990 removed understory vegetation and may have enhanced mourning dove populations the following spring, contributing to SLE amplification during the 1990 epidemic year (Day *et al.*, 1999).

F. Weather

Climate variability affects weather patterns and therefore SLE transmission at three scales: 1) long-range (decadal time scale) global warming may increase the length of the transmission season and

extend the geographical range of SLE (Reeves *et al.*, 1994); 2) annual changes (intradecadal) based on El Niño/southern oscillation (ENSO) changes in the Pacific sea surface temperature markedly alter precipitation and temperature patterns over the Americas that cycle with varying intensity at 3- to 5-year intervals (Glantz, 2001; Gubler *et al.*, 2001); and 3) monthly changes (intra-annual) based on the relationship of the jet stream to ENSO patterns in the Pacific markedly alter day-to-day weather regionally and alter the efficiency of SLE amplification. Scales 2 and 3 alter storm tracks and thereby change the intensity and direction of hurricanes forming in the Caribbean and Atlantic and where they impact the southern United States. In addition to temperature, weather alters precipitation and water availability and can influence SLE risk in several ways:

1. Vector Abundance

Precipitation in the form of winter rainfall and snow pack in the west (Wegbreit and Reisen, 2000) or summer rainfall in the east (Day and Curtis, 1989) creates oviposition and larval habitats for vector species. Rural species such as *Cx. tarsalis* and *Cx. nigripalpus* that breed in surface pools increase in abundance as rainfall creates new habitats (Day *et al.*, 1993; Reisen *et al.*, 1990). In contrast, urban species such as the *Cx. pipiens* complex that exploit municipal drainage systems increase in abundance when rainfall decreases and drainage systems dry, creating pooling. Droughts that increase *Cx. pipiens* complex abundance have been associated with SLE epidemics in St. Louis (Lumsden, 1958) and Delaware (Altman and Goldfield, 1968).

2. Humidity

The pattern of rainfall as well as the quantity may influence vector host-seeking behavior and therefore transmission. In general, elevated humidity tends to increase vector survival enhancing transmission efficiency. Also, tropical vectors such as *Cx. nigripalpus* blood feed only after humidity increases following rainfall (Day et al., 1989). During dry periods, host-seeking is arrested and virus infections in gravid and parous vectors complete extrinsic incubation, resulting in the accumulation of infective females; rainfall increases humidity, releasing host-seeking behavior and results in synchronized transmission events.

3. Avian Abundance

Rainfall patterns affect both plant and insect food availability and thereby influence bird survival, nesting success, number of broods per year, and population size and age structure. Although these factors

are critical for virus transmission, their impact may be delayed until the following season.

VI. Transmission Cycles

Birds in the orders Passeriformes and Columbiformes seem to be the principal summer vertebrate hosts and mosquitoes within the genus *Culex*, the principal vectors of SLE virus in North America (Fig. 4). However, the ecology of this basic cycle varies regionally, depending on the biology, vectorial capacity and vector competence of regional mosquito species, virulence of viral strains, and competence of regional vertebrate hosts. Seasonal transmission activity may be divided into overwintering, vernal and/or summer amplification, and autumnal subsidence periods.

A. *Overwintering*

Despite over 50 years of intensive investigation on encephalitis virus persistence, the primary mechanism(s) remains essentially unknown, although some data exist to support three very different mechanisms.

1. Persistence in Mosquito Populations

Winter isolation of virus from mosquitoes and early season detection of SLE infection in sentinel chickens support the concept of virus persistence within local vector populations. Three mechanisms

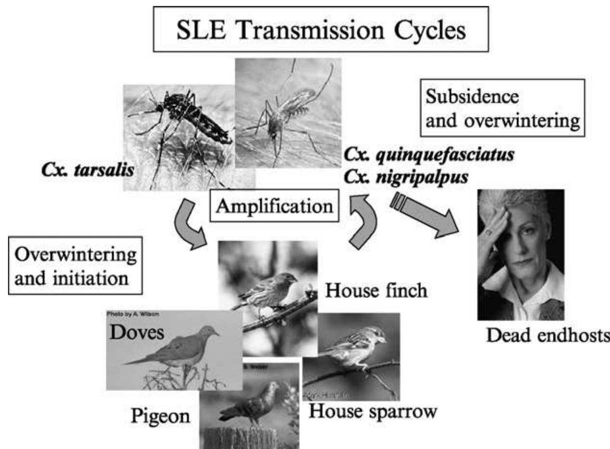


Fig 4. Summer transmission cycle of SLE virus in North America.

(vertical transmission, gonotrophic dissociation, and continued horizontal transmission) have been described that might explain SLE persistence within vector populations within the United States.

1. Vertical transmission. Low level vertical passage of SLE virus from infected females to F1 progeny has been demonstrated experimentally for several mosquitoes, including the vectors *Cx. p. quinquefasciatus*, *Cx. tarsalis*, and *Cx. nigripalpus* (Chamberlain *et al*., 1964; Francy *et al*., 1981; Hardy *et al*., 1984; Nayar *et al*., 1986). Vertical transmission was most efficient when larvae were raised under cool temperatures (Hardy *et al*., 1980), similar to those experienced by larvae in nature during fall and spring. However, vertical transmission remains undocumented in nature either by virus isolation from adults reared from field-collected immatures or from adult males (Reeves, 1990b; Reisen *et al*., 1992b, 1992c, 2002b). In contrast, vertical transmission has been documented in nature for other flaviviruses within the JE serocomplex, including JE (Dhanda *et al*., 1989; Rosen *et al*., 1980) and West Nile (WN) viruses (Miller *et al*., 2000; Nasci *et al*., 2001).

2. Gonotrophic dissociation. Female *Cx. p. pipiens* females destined for diapause have been shown to take small blood meals during late summer and early fall without developing their ovaries (somewhat similar to gonotrophic dissociation in *Anopheles*) (Eldridge, 1968). Two isolations of SLE virus were made from diapausing *Cx. p. pipiens* females collected resting during winter in Maryland that were considered to have been infected by this mechanism (Bailey *et al*., 1978), although infection by vertical transmission also was possible. Experimentally, *Cx. p. pipiens* and *Cx. tarsalis* infected by this mechanism were found to overwinter and then transmit virus (Eldridge and Bailey, 1979; Reisen *et al*., 2002a). However, *Culex* females in reproductive diapause were reluctant to take blood meals (Mitchell, 1981; Mitchell and Briegel, 1989), and nulliparous females rarely were collected host-seeking during autumn (Nelson, 1964), questioning the occurrence of this infection mechanism in naturally diapausing mosquitoes.

3. Horizontal transmission. *Culex p. quinquefasciatus* does not enter reproductive diapause, remains reproductively active throughout winter at southern latitudes (Hayes, 1975) and, depending on ambient temperature, could maintain SLE virus by continued, infrequent transmission among resident birds. Experimentally infected, reproductively active females have been shown to survive winter as gravid females and then transmit SLE virus to recipient birds the following spring in

California (Bellamy *et al.*, 1968). This mechanism may allow SLE persistence at southern latitudes where *Cx. p. quinquefasciatus* is the primary vector, but its role in areas such as California and Florida seems doubtful.

2. Persistence in Vertebrate Populations

SLE virus also may persist over winter within vertebrate host populations. Recent research has shown that passeriform birds infrequently develop chronic infections that persist as long as a year after experimental infection with SLE (Reisen *et al.*, 2001b). However, attempts to demonstrate natural relapse by detecting a peripheral viremia (Reisen *et al.*, 2001b) or by vector infection (Reeves, 1990b) have not been successful, even following the experimental suppression of host immunocompetence (McLean *et al.*, 1983; Reisen *et al.*, 2001b). Immunosuppression triggered relapses of cryptic avian malaria infections, but not concurrent SLE infections (Reisen *et al.*, 2001b).

Flaviviruses, including SLE, have been isolated repeatedly from bats (Sulkin *et al.*, 1974). Mexican free-tailed bats (*Tadardia brasiliensis*) are susceptible to experimental infection with SLE virus (Sulkin *et al.*, 1966), and infections in bats destined for hibernation have been maintained for 20 days at 10 °C. When returned to room temperature, SLE virus was detected in the brown fat of seven of 11 bats and at low levels in the blood of two bats (Allen *et al.*, 1970). These data indicated that bats could function as an overwintering host. However, surveys of mosquito host selection patterns indicated that bats rarely, if ever, were fed upon by host-seeking mosquitoes (Reisen *et al.*, 1990; Tempelis, 1975; Washino and Tempelis, 1983). Recently, house finches have been shown experimentally to become infected with WN virus after feeding on infected mosquitos (Komar *et al.,* 2003), and perhaps it is the frequent feeding by insectivorous bats on infected mosquitoes that resulted in these bat infections (Allen *et al.*, 1970).

As reviewed previously, a variety of mammals other than bats are competent hosts (McLean *et al.*, 1985a) and the tick *Dermacentor variabilis* is a competent laboratory vector of SLE virus (McLean *et al.*, 1985b). In addition, SLE virus was isolated from an adult *D. variabilis* removed from a raccoon that was not viremic (McLean *et al.*, 1985b). These data indicated that an alternate cycle among mammals and ticks might be possible. Movement of SLE virus into birds by ticks may be feasible because ticks carrying a variety of other mammalian pathogens have been collected from birds (Alekseev *et al.*, 2001; Scott *et al.*, 2001a). However, demonstration of transtadial or vertical transmission of SLE

virus in ticks would seem necessary for virus to be maintained in a tick-mammal cycle and then jump to the primary *Culex*-bird cycle. In addition, ticks rarely are observed on birds collected in the desert regions of California where SLE appears during most summers.

3. Re-introduction of virus

An alternative hypothesis to local persistence involves annual or periodic extinction and re-introduction. Long distance movement of SLE virus has been indicated indirectly from genetic evidence (Kramer *et al.*, 2001) as well as by the re-appearance of SLE after years of absence. Two possible hypotheses address re-introduction, but neither is well supported by field evidence.

a. Migratory birds or bats Many species of birds and some bats have long distance annual migrations that could allow the transport of virus from foci active during winter in southern latitudes or south of the equator to receptive areas north of the equator during spring. These vertebrate populations typically are very consistent in their summer and winter destinations, and this would allow the same or similar genetic strains to re-appear each summer at the same locality. However, migration destinations are not fixed. The possible introduction of WN virus into the Western Hemisphere by migratory birds recently has been reviewed (Rappole *et al.*, 2000). In addition, some bird species exchange flyways (i.e., birds moving south in one flyway return north in a different flyway), and this could allow the movement of SLE strains into other regions (Kramer *et al.*, 1997). However, North, Central, and South American isolates are distinct genetically, implying infrequent exchange (Kramer *et al.*, 2001). In addition, migratory bird species do not seem to be frequently involved in transmission and infrequently are positive for virus (Calisher *et al.*, 1971) or antibody (Reisen *et al.*, 2000).

b. Wind-dispersed mosquitoes Strong updrafts associated with storm fronts or prevailing weather patterns can move vectors long distances as “aerial plankton,” and these storm fronts have been suggested as methods for vector and virus dispersal (Sellers and Maarouf, 1990), including the movement of JE into northern Australia from Papua New Guinea (Hanna *et al.*, 1999; Kay and Farrow, 2000; Ritchie and Rochester, 2001). However, the prevailing west-to-east storm patterns in North America (Glantz, 2001) would seem counterintuitive to virus introduction from Mexico or Central America and are the wrong direction for the recorded westerly spread of WN virus.

B. Amplification

Regardless of the mechanism of persistence or introduction, once virus amplification is initiated, the primary transmission cycles of SLE during summer in North America involve mosquitoes in the subgenus *Culex* of the genus *Culex* and birds in the orders Passeriformes and Columbiformes (Fig. 4). Occasional isolations have been made from other mosquito taxa, and some of these incidental hosts have been shown to be competent laboratory vectors (Mitchell *et al.*, 1980a); however, few of these incidental hosts feed frequently on birds and therefore are not considered important in viral amplification. Transmission appears to be initiated after *Cx. tarsalis* and *Cx. p. pipiens* terminate diapause or *Cx. p. quinquefasciatus* and *Cx. nigripalpus* terminate quiescence, and ambient temperatures warm sufficiently to allow the rapid replication of virus in the mosquito host. Virus imbibed during infectious blood meals taken in early in spring when ambient conditions average $<17\,^{\circ}C$ may lay dormant until warm conditions or host mosquito physiology stimulates replication (Bailey *et al.*, 1978; Reisen *et al.*, 2002a).

Most of the transmission cycle is spent within the mosquito host that is infected for life. Transmission is horizontal and biologic; i.e., infection is acquired when a female *Culex* blood feeds on a viremic avian host, virus is amplified and disseminated within the mosquito during the extrinsic incubation period, and then transmitted after the female oviposits and takes a subsequent blood meal. The quantity of virus present in the peripheral bloodstream of most viremic hosts and therefore potentially present within the proboscis of feeding females would seem to be insufficient for mechanical transmission. In addition, partial blood meals due to interrupted feeding are a rare occurrence in *Culex* vectors, such as *Cx. tarsalis* (Mitchell *et al.*, 1981). Biological transmission requires more than 10 days and perhaps two mosquito gonotrophic cycles when ambient temperatures average $22\,^{\circ}C$ (Reisen *et al.*, 1993b). In contrast, the viremia response in susceptible avian hosts typically is short, lasting 2–4 days after infection (McLean *et al.*, 1980).

The four geographically distinct transmission cycles of SLE virus are defined by differences in the biology of the primary vector mosquito species, and include: 1) rural North America, west of the Mississippi River transmitted by *Cx. tarsalis*; 2) rural and urban central and eastern North America transmitted by members of the *Cx. pipiens* complex; 3) Florida, Caribbean, and parts of Central America transmitted by *Cx. nigripalpus*; and 4) urban and rural South America

transmitted by *Cx. pipiens* complex and other mosquito taxa (Table I). Interestingly, the distribution of the principal mosquito vectors (Figs. 5, 6) and the principal avian hosts (Fig. 7) overlap, but it is unclear why transmission cycles involving selected vector and vertebrate species predominate regionally or why they have led to the evolution of different levels of SLE virulence. For example, *Cx. p. quinquefasciatus* is considered to be the primary vector in urban Texas and the southeast, but not in the southwest or Florida, where it also is regionally abundant (Darsie and Ward, 1981). Similarly, *Cx. tarsalis* is distributed throughout the southern United States, but is not abundant or considered to be an important vector east of the Mississippi.

In western North America, *Cx. tarsalis* is the primary vector and house finches the primary avian host (Reeves, 1990a; Reeves *et al.*, 1962). Enzootic transmission with few human cases currently occurs annually in irrigated southwestern agrarian settings such as the Imperial and Coachella Valleys of California (Reisen *et al.*, 1992a), whereas SLE epidemics in the 1980s were associated with suburban/urban settings in Bakersfield and Los Angeles, California (Murray *et al.*, 1985; Tueller, 1990). After amplification within the primary *Cx. tarsalis*-bird cycle, other mosquito species including *Cx. p. quinquefasciatus* and *Cx. stigmatosoma* may become involved, especially in suburban/urban environments. *Cx. tarsalis* feeds most frequently upon passeriform birds

TABLE I
COMPONENTS OF FOUR SLE TRANSMISSION CYCLES IN THE NEW WORLD

Geographical cycle area	Vectors	Avian hosts
1. Western North America	*Cx. tarsalis*	House finches
	Cx. quinquefasciatus	Mourning doves
	Cx. stigmatosoma	
2. Central and Eastern North America	*Cx. quinquefasciatus*	House sparrows
	Cx. pipiens	Mourning doves
	Cx. restuans	Pigeons
	Cx. salinarius	Blue jays
3. Florida	*Cx. nigripalpus*	Mourning doves
	Cx. quinquefasciatus?	Grackles
		House sparrows
4. Central and South America	*Cx. quinquefasciatus*	Variety of bird species
	Cx. nigripalpus	Rodents
	Culex spp.	Sloths?
	Other mosquitoes	

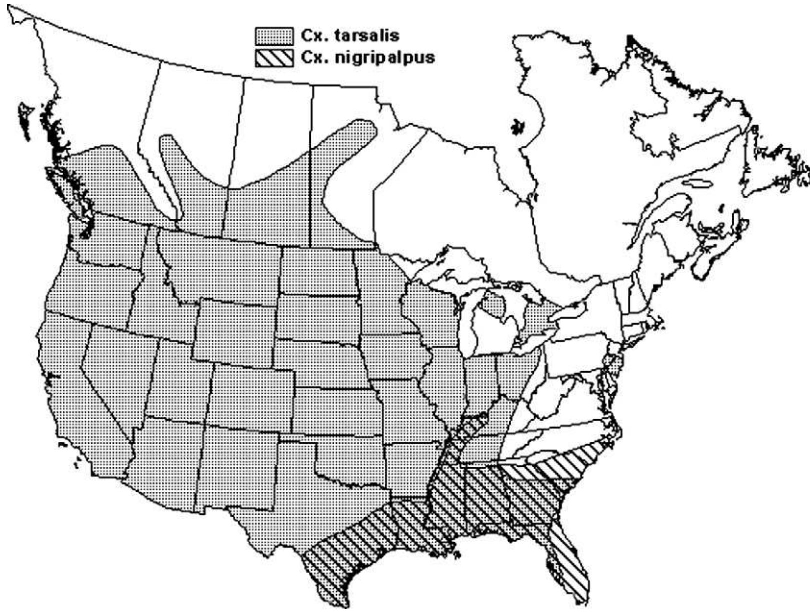


FIG 5. Geographical distribution of *Culex tarsalis* and *Cx. nigripalpus* in North America north of Mexico. (Redrawn from Darsie and Ward, 1981).

during late winter and early spring, but expands its host range to include other birds as well as mammals including humans as population size increases (Tempelis *et al.*, 1965). This host-feeding shift relates to the comparative tolerance of avian and mammalian hosts to mosquito biting and avoidance behavior in response to the biting density of mosquitoes (Nelson *et al.*, 1976). The shift in host selection occurs during midsummer in the Central Valley of California (Tempelis *et al.*, 1967) and elsewhere in the West (Reeves, 1971), but may occur earlier at lower latitudes such as southeastern California, where population abundance peaks during spring (Lothrop and Reisen, 2001). In the Los Angeles basin, *Cx. p. quinquefasciatus* is extremely abundant, feeds frequently on both birds and mammals, and could be important as a bridge vector transmitting infections acquired from birds to humans (Reisen *et al.*, 1992c). In contrast, *Cx. stigmatosoma* feeds almost exclusively on birds (Reisen *et al.*, 1990) and therefore may be important in amplifying SLE within the urban avian enzootic cycle. These three species differ considerably in their vectorial capacity and competence for western strains of SLE, with *Cx. stigmatosoma* most susceptible to oral infection, followed by *Cx. tarsalis* and *Cx. p.*

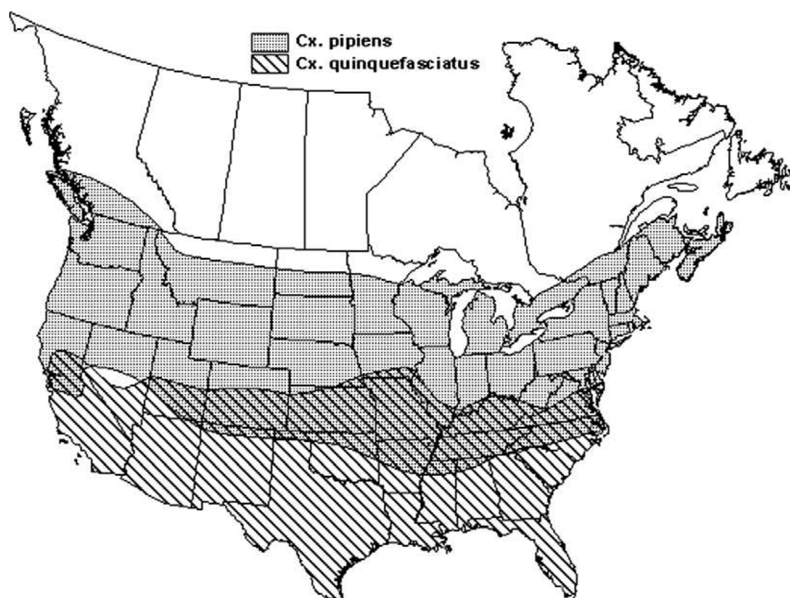
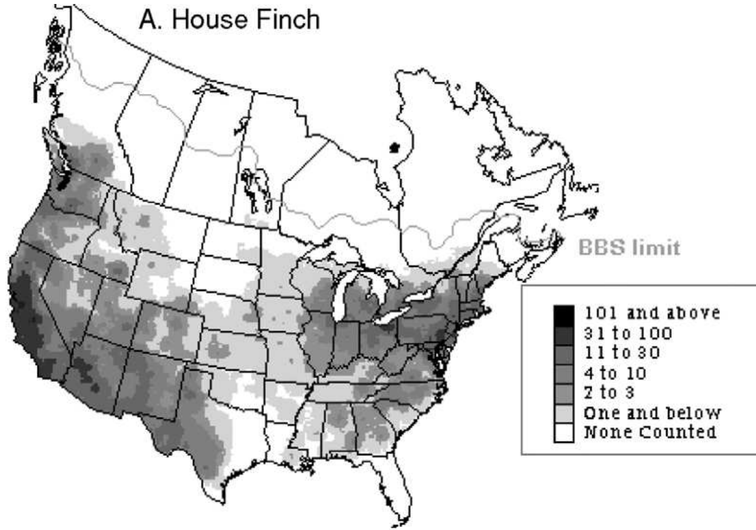
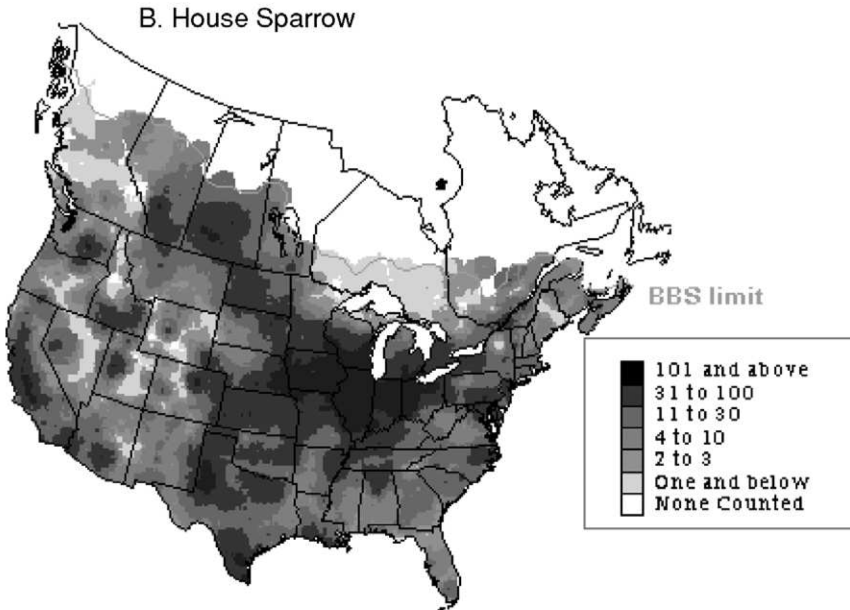


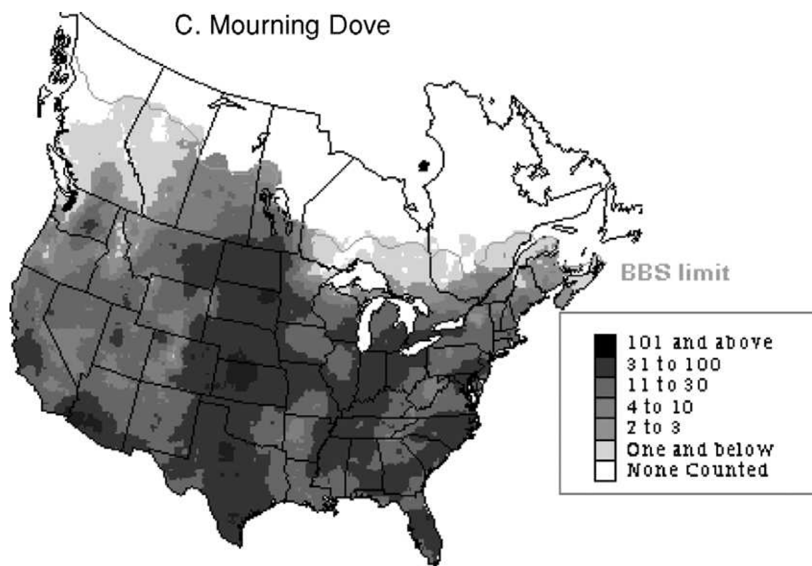
FIG 6. Geographical distribution of the *Culex pipiens* complex in North America north of Mexico. (Redrawn from Darsie and Ward, 1981).

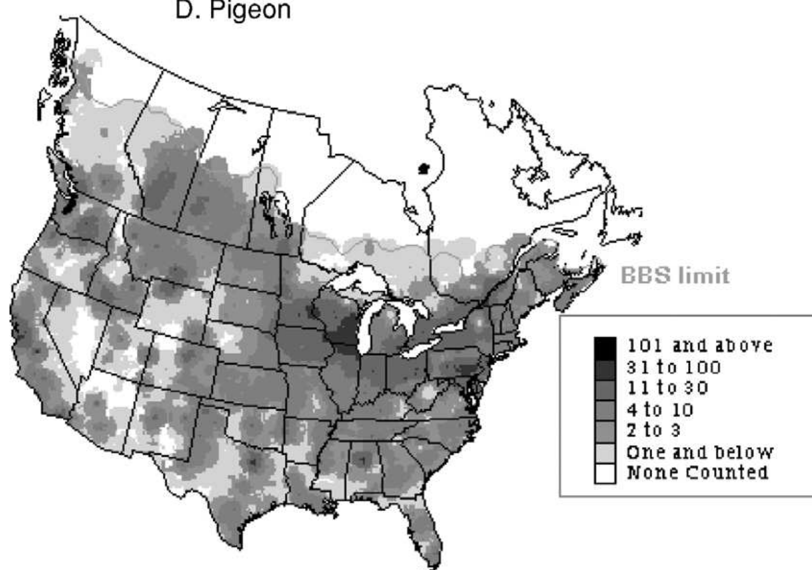
quinquefasciatus (Meyer *et al.*, 1983; Hardy *et al.*, 1986, 1990a). Susceptibility seems to be related to a gut (mesenteronal) infection or escape barrier, because most infected females with disseminated infections transmit virus per os. Avian host competence studies using recent isolates of SLE from California have found that adults of few species other than house finches and song sparrows consistently produce peripheral viremias of sufficient titer to infect mosquitoes (Reisen *et al.*, 2001a), perhaps indicating the importance of nestlings in virus amplification. Vectorial capacity (measured as infection dissemination among birds) has been estimated in Los Angeles for the three *Culex* vectors (Reisen *et al.*, 1992c). Although all estimates were low, *Cx. tarsalis* was the best vector, followed by *Cx. quinquefasciatus* and then *Cx. stigmatosoma*.

From the Midwest through the Atlantic states, members of the *Culex pipiens* complex, including *pipiens*, *quinquefasciatus*, and perhaps *molestus* (an autogenous subtype of *pipiens* mostly recognized in the East) are the primary vector(s), and house sparrows, mourning doves, pigeons, and blue jays are the primary avian hosts (Monath, 1980a). Within the past decade, house finches have become established in the


A. House Finch
BBS limit
101 and above
31 to 100
11 to 30
4 to 10
2 to 3
One and below
None Counted


B. House Sparrow
BBS limit
101 and above
31 to 100
11 to 30
4 to 10
2 to 3
One and below
None Counted


C. Mourning Dove
BBS limit
101 and above
31 to 100
11 to 30
4 to 10
2 to 3
One and below
None Counted


D. Pigeon
BBS limit
101 and above
31 to 100
11 to 30
4 to 10
2 to 3
One and below
None Counted

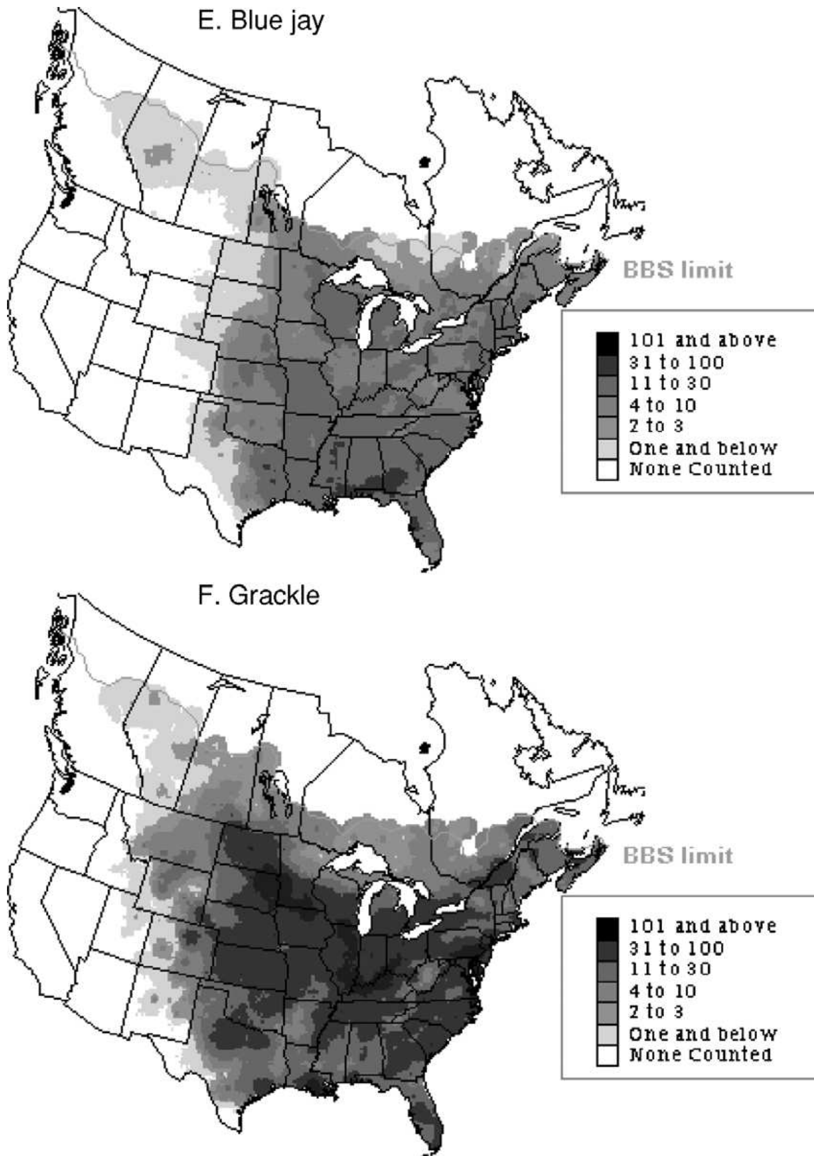


FIG 7. Summer distribution of avian hosts of SLE in North America north of Mexico based on summer breeding bird survey data from 1966–2000. (Maps from Sauer, J. R., J. E. Hines, J. Fallon. (2001). The North American Breeding Bird Survey, Results and Analysis 1966–2000. Version 2001.2, USGS Patuxent Wildlife Research Center, Laurel, MD. Available at:www.mbr-pwrc.usgs.gov/bbs/bbs.html)

Northeast and these populations seem to be expanding (Fig. 7). If they become abundant in the South, they may provide another important avian host for SLE in this region. Temperature may determine the distribution of the *Culex pipiens* complex in North America (Barr, 1957). Based on morphology of the male terminalia, *Cx. p. pipiens* is found north of 39 °N latitude and *Cx. p. quinquefasciatus* is found south of 36 °N, with intergrades between 36 and 39 °N. However, this distribution actually may be less clearly delineated, because subsequent population genetic studies based on isozymes (Tabachnick and Powell, 1983; Urbanelli *et al.*, 1997) have shown considerable introgression between terminalia types. Although their vector competence for SLE appears to be similar (Chamberlain *et al.*, 1959, 1966; Hardy *et al.*, 1990b), subspecies within the *pipiens* complex exhibit important biological differences. *Cx. p. pipiens* exhibits facultative autogeny (Spielman, 1971), enters reproductive diapause in late summer (Eldridge, 1968; Spielman and Wong, 1973), and blood feeds primarily on avian hosts (Tempelis, 1975), whereas *Cx. p. quinquefasciatus* is anautogenous, remains reproductively active throughout the year (Hayes, 1975), and blood feeds on both mammalian and avian hosts (Tempelis, 1975). These differences markedly alter the ecology of virus transmission by delineating the length of the transmission season and the frequency of human contact. Additional *Culex* species such as *Cx. restuans* and *Cx. salinarius* also may be important in SLE amplification, because they feed on avian hosts (Washino *et al.*, 1983), are susceptible to infection (Chamberlain *et al.*, 1959), and multiple isolations have been made during outbreaks in Tennessee and Illinois (Mitchell *et al.*, 1980a). Coevolution between SLE and members of the *Cx. pipiens* complex that are relatively less susceptible to infection than other *Culex* vectors (Meyer *et al.*, 1983; Mitchell *et al.*, 1980a) may have led to the selection for SLE strains in the east that are more virulent in mice (Monath *et al.*, 1980b) and viremogenic in avian hosts (Bowen *et al.*, 1980) than are SLE strains from the west.

In Florida and parts of tropical America, *Cx. nigripalpus* appears to be the primary enzootic and epidemic vector. Comparable to *Cx. tarsalis* in the West, *Cx. nigripalpus* is a highly competent experimental vector of SLE (Chamberlain *et al.*, 1966; Sudia and Chamberlain, 1964) from which virus frequently was isolated during outbreaks in Florida during 1962 (Dow *et al.*, 1964), 1977 (Nelson *et al.*, 1983) and 1990 (Shroyer, 1991). Although *Cx. p. quinquefasciatus* also is abundant throughout Florida (Darsie *et al.*, 1981), this species is not considered to be an important primary or secondary vector. *Cx. nigripalpus* feeds predominantly on avian hosts, but seasonally

extends this pattern to include mammals and humans (Edman and Taylor, 1968). In addition to warm summer temperature required for efficient virus extrinsic incubation, intermittent rainfall events synchronize oviposition, blood feeding and therefore transmission by parous and infective females (Day *et al.*, 1990). The pattern and amplitude of these rainfall events determine the number of annual broods of *Cx. nigripalpus* and therefore the magnitude of summer population size (Day *et al.*, 1993).

Far less is known about SLE transmission cycle(s) south of the United States (Spence, 1980). In Central and South America, SLE virus has been isolated from 11 different genera of mosquitoes, including the recognized northern vectors *Cx. nigripalpus* (Aitken *et al.*, 1964; Belle *et al.*, 1964) and *Cx. p. quinquefasciatus* (Mitchell *et al.*, 1980b). SLE virus also has been isolated from a wide variety of bird taxa, including herons, egrets, and cormorants (Spence, 1980). Interestingly, many of these tropical mosquito hosts feed most frequently on mammals, and a variety of forest mammals also have been found infected. These data, coupled with marked genetic differences between North and South American strains of SLE (Kramer *et al.*, 2001), indicate there may be significant differences in the ecology and epidemiology of SLE in tropical America, a topic requiring additional field research.

Seroprevalence surveys during summer typically indicate that wild birds fall into three general categories, due to the interaction between roosting/nesting locations and mosquito host-seeking behavior (Lothrop *et al.*, 2001): 1) frequently infected, 2) occasionally infected, and 3) never infected. At an enzootic SLE focus in southern California where 10,945 sera were tested for SLE antibody over a 3-year period, four species (house finch, house sparrow, Gambel's quail, and common ground dove) fell into category 1 (>80% of total serologically positive), five into category 2, and 115 into category 3 (Reisen *et al.*, 2000). All of the category 1 and 2 birds were resident species, whereas many of category 3 species were migrants or winter residents. The product of infection prevalence multiplied by percentage composition of the total avian fauna has been used to express the relative contribution of different bird species to transmission (Lord *et al.*, 1974b). Using this approach, house finches were the most important species in California, mourning doves in Florida, and house sparrows in Texas (McLean *et al.*, 1980).

Host competence information is necessary to interpret seroprevalence data and can be expressed as the number of days that an avian host produces a viremia of sufficient titer to infect the primary mosquito vector species (Hardy *et al.*, 1990a; Komar *et al.*, 1999). Host

competence varied considerably as a function of virus strain (Bowen *et al.*, 1980) and infectious dose (McLean *et al.*, 1980). Recent studies using SLE strains from California have shown that at doses normally expectorated by *Cx. tarsalis* females, few species of adult birds become viremic (Reisen *et al.*, 2001a), perhaps emphasizing the importance of nestling birds in virus amplification.

C. Subsidence

Enzootic transmission intensity and the detection of new human cases always subsides during fall in North America. Cool temperatures slow the replication of SLE in mosquito hosts, decreasing the efficiency of transmission, and concurrently the combination of cooling water temperatures and shortening days during larval development initiates reproductive diapause (*Cx. tarsalis, Cx. p. pipiens*) or quiescence (*Cx. p. quinquefasciatus, Cx. nigripalpus*) in vector females emerging during fall. The fall mosquito population bifurcates into newly emerged females destined for diapause that do not routinely blood feed and remnants of the summer population that continue reproductive activity, but fail to survive winter (Nelson, 1964). Continued blood feeding by this aging population may result in late season transmission and seroconversions by sentinel chickens have been detected as late as December in Los Angeles (Emmons *et al.*, 1988). *Cx. tarsalis* diapause is maintained by shortening photoperiods through the winter solstice; thereafter, the termination date becomes progressively later moving northward and is determined by degree-day accrual (Reisen *et al.*, 1995). Consequently, females initiate host-seeking in late December in southern California (Reisen *et al.*, 1995), in mid-January in the San Joaquin Valley of California (Bellamy *et al.*, 1963; Reisen *et al.*, 1986), but in April in Colorado (Mitchell, 1979). Termination date has been forecasted by degree-day models using soil temperatures (Bennington *et al.*, 1958). The critical day length that triggers the onset of diapause in *Cx. p. pipiens* may occur in late summer at northern latitudes (Spielman *et al.*, 1973), markedly shortening the SLE transmission season. During warm days, however, females may become infected when taking partial blood meals from viremic birds (Bailey *et al.*, 1978), survive winter (Bailey *et al.*, 1982), and then transmit this infection after diapause is terminated by warm spring temperatures. *Cx. p. quinquefasciatus* does not undergo diapause (Reisen *et al.*, 1986) and reproductive activity may continue through winter, albeit at a rate slowed by winter temperatures (Hayes, 1975). Gravid and infected *Cx. p. quinquefasciatus* females that survive winter under cool

temperatures at southern latitudes are capable of transmission the following spring (Bellamy *et al.*, 1968).

VII. Surveillance and Control

Effective vector control remains the only intervention approach available to suppress summer virus amplification and prevent human infections. Protection of the human population by vaccination does not seem cost effective or prudent, because there is no human-to-human transmission, few human infections produce disease, and infection rates remain relatively low, even during epidemics. However, if regional infection rates were to become high, thereby placing selected cohorts at high risk of disease, then selective vaccination may be warranted. Vaccination of school children against JE is done in high-risk rural areas of Japan. There currently is no approved commercial vaccine for SLE, although vaccination against other flaviviruses may impart some protection (Hammon *et al.*, 1966). Control of avian hosts such as house sparrows and pigeons in urban situations could be done, but this approach is not generally acceptable to the public. Notification of the public of infection risk through the media and the wide scale use of personal protection through changes in behavior (staying indoors after sunset) and/or repellent application were credited with reducing the numbers of infections during the 1990 epidemic in Florida (Meehan *et al.*, 2000).

An effective surveillance program is the key to planning, implementation, and evaluation of vector control. Surveillance programs designed for SLE virus and other mosquito-borne encephalitis viruses typically integrate information on weather, mosquito abundance and infection rates, free ranging avian and/or sentinel infection rates, and human cases (Eldridge, 1987; Reeves *et al.*, 1990). Although the objective of these programs is to forecast infection in humans and thereby intercede to prevent their occurrence, forecasting skill decreases as a function of time preceding the transmission event. Recent focus on climate variability caused by changes in sea surface temperatures in the Pacific altering the Walker oscillation in rain cloud formation has led to the development of global circulation models to predict ENSO events. Because there are strong links between ENSO events and temperature and precipitation patterns in the New World (Glantz, 2001), these global models may be useful in forecasting weather 6 months to 1 year in advance, thereby allowing health planners sufficient time to anticipate high- or low-risk years for SLE activity. Years

with above-normal spring and summer temperatures should be considered at risk for increased SLE activity. As a forecasting model in Kern County, California, *Cx. tarsalis* abundance and western equine encephalomyelitis (WEE) virus risk during summer increased when snow pack in the Sierra Nevadas during the preceding spring and subsequent Kern River run-off increased (Reeves *et al.*, 1962; Wegbreit *et al.*, 2000), allowing fairly long-term forecasting. In Florida, the number and frequency of rainfall events >2.5 cm within 72 hours were related closely to *Cx. nigripalpus* population size and transmission, whereas freezes that killed understory vegetation enhanced avian populations and contributed to rapid amplification (Day, 2001).

The intensity of SLE enzootic transmission and the risk of tangential transmission to humans have been related to *Culex* population size (Olson *et al.*, 1979) that may be measured by a variety of methods. *Cx. tarsalis* is attracted to lights, and populations have been monitored for years in the west using NJ light traps (Mulhern, 1942), although dry ice–baited Centers for Disease Control–style traps (Newhouse *et al.*, 1966) always collect more specimens and therefore are a more sensitive indicator of population change (Reisen *et al.*, 1999). *Culex pipiens* complex females are not attracted to lights, but can be collected by dry ice–baited traps (Reisen *et al.*, 1999). Gravid female traps (Reiter, 1983) are the most sensitive method of collecting *Cx. pipiens*; however, effective attractants have yet to be discovered for *Cx. tarsalis* (Reisen *et al.*, 1999) or *Cx. nigripalpus*. *Culex nigripalpus* appears to be difficult to sample, and recent studies have emphasized the mechanical aspiration of vegetation at resting sites (Day and Edman, 1988).

Because mosquito virus infection rates typically are very low, specimens usually are tested in pools consisting of 25–50 females, and infection incidences are expressed as minimum infection rates (MIR) per 1000 females tested per interval (Chiang and Reeves, 1962). By definition, the range of possible infection rates is delineated by pool size; however, MIRs exceeding one per 1000 (one positive pool per 20 pools of 50 females tested) are considered to be of public health significance (Day, 2001). These are infection and not transmission rates. Studies using seroconversion rates among young chickens as bait in lard can traps indicated that approximately one in four infected host-seeking *Cx. tarsalis* females were capable of transmission (Reeves *et al.*, 1961).

Seroprevalence rates in free-ranging birds collected by traps or nets have been used to monitor SLE transmission activity (Day *et al.*, 1999; Gruwell *et al.*, 1988; Lord *et al.*, 1974a). Sampling that targets critical species such as house sparrows (McLean *et al.*, 1983) is

generally more efficient than programs sampling the entire avian fauna (Reisen *et al.*, 2000). Extensive collection effort, difficulties testing sera from a variety of avian species, problems with aging specimens, and difficulty determining the place of infection confound the interpretation of data obtained from wild bird sampling and limit their utility in control programs (Reisen *et al.*, 2000). Historically, farm chickens were infected frequently during serosurveys (Hammon *et al.*, 1943) and later were used as sentinels to monitor transmission (Reeves *et al.*, 1990). Small flocks of five to 10 chickens provide a sensitive and cost-effective method to monitor SLE transmission activity in the West (Reisen *et al.*, 2000; Scott *et al.*, 2001b) and in Florida (Day, 1989), where *Cx. tarsalis* and *Cx. nigripalpus* are primary vectors, respectively. Sentinel chickens seem less effective as sentinels in urban areas where *Cx. pipiens* complex females are the primary vectors. For example, surveillance in Houston, Texas, relies mostly on monitoring virus infection rates in mosquitoes (Chandler *et al.*, 2001). These differences in the sensitivity of sentinel chickens to monitor infection transmitted by different vectors have come to the forefront during the recent WN outbreak (Langevin *et al.*, 2001). Adult chickens are especially suitable as sentinels, because they can be purchased inexpensively in large numbers, do not develop viremias of sufficient titer to infect mosquitoes, and do develop high titered and long-lasting antibody (Reisen *et al.*, 1994). In California, blood samples from >2000 sentinel hens distributed throughout the state are collected biweekly by lancet prick of the comb onto filter paper and tested by EIA, precluding the need for invasive sampling with needles, specimen clarification, by centrifugation, and maintenance of a cold chain (Reisen *et al.*, 1993a).

Early recognition of human infection with SLE virus is critical because it indicates that enzootic amplification has reached levels where the virus has spilled over into the human population. Typically, cases are recognized by a passive surveillance system that relies on the primary health care system to provide case recognition, reporting and proper specimens for definitive diagnosis. Sharing information on increased enzootic amplification in the form of medical alerts may be critical in raising the awareness of the medical community to take appropriate blood samples for serologic diagnosis. Active monitoring of high-risk segments of the human population such as the elderly, visitors to homeless shelters, and rural farm workers may enhance sensitivity and provide an early warning of human involvement. Confirmed human involvement escalates intervention by mosquito control agencies from routine to emergency levels to remove infected female mosquitoes from areas of human contact.

VIII. Impact of West Nile Virus Introduction

The recent invasion of North America by WN virus has stimulated new interest in arbovirology and provided an unprecedented rebuilding of surveillance and control programs (Holloway, 2000). SLE and WN viruses are closely related antigenically and classified within the Japanese encephalitis (JE) serocomplex (Calisher *et al.*, 1989). Most members of this complex are allopatric in their distribution (Work, 1971), although WN is sympatric with JE in central India through eastern Pakistan, and Murray Valley encephalitis virus is sympatric with Kunjin virus in Australia (Kuno *et al.*, 1998). The 2001 invasion of areas in southeastern North America endemic for SLE by WN will provide new opportunities to study coactions between these viruses and will greatly complicate laboratory diagnostics in these areas.

Presumably the most intense coactions between these viruses will be associated with the immune structure of avian and other vertebrate host populations. WN infection is especially lethal to corvids (Eidson *et al.*, 2001), produces elevated viremias in a wide variety of birds (Komar, 2003), and elicits a protective antibody response (Hayes, 1989). Although less virulent and viremogenic, SLE infection also produces lasting immunity (McLean *et al.*, 1983). Acquired immunity to either virus in birds is expected to provide considerable cross protection and prevent re-infection, similar to cross protection observed in humans among other flaviviruses (Bond and Hammon, 1970) and demonstrated experimentally in hamsters (Tesh *et al.*, 2002). At maintenance transmission levels, seroprevalence rates within avian host populations typically remain low (Day *et al.*, 1999; Reisen *et al.*, 2000) and would provide little resistance to introduction and amplification by a second virus. However, widespread SLE infection in birds associated with disease in humans frequently results in seroprevalence rates in resident bird species >50% (McLean *et al.*, 1980). The acquisition of "herd immunity" at this level should dampen the amplification of a second virus during the same transmission season and perhaps both viruses during the subsequent year. Protective immunity in house sparrows was demonstrated to persist for 2 years (McLean *et al.*, 1983), verifying long-term protection. In humans, cross protection from previous dengue infection and yellow fever immunization was investigated during the course of SLE epidemics in Florida (Bond *et al.*, 1970; Hammon *et al.*, 1966), and acquired immunity from previous SLE infection similarly may afford protection from WN. Protection of WN disease by previous infection with SLE has been demonstrated recently using a hamster model (Tesh *et al.*, 2002). Acquired protection could

be especially important in epidemics, because similar to SLE, cohorts >65 years of age have the greatest risk of clinical disease from infection with WN. Immunity appears to be retained over time and therefore population immunity would increase cumulatively with cohort age. The oldest cohort at highest risk of illness also should have the greatest chance of having acquired protective immunity.

The introduction of WN into SLE endemic areas will seriously impact laboratory diagnostics that rely on serology (Calisher *et al.*, 1989) and thereby compromise surveillance information (Tesh *et al.*, 2002). The initial human cases of WN in New York, for example, were diagnosed as SLE (CDC, 1999), and even after WN was confirmed from necropsy material, the serological picture was not clear in all cases. In birds and horses, flavivirus neutralizing antibody titers range from low to moderate, thereby possibly compromising the use of tissue culture plaque reduction neutralization (PRNT) end point titrations as a confirmatory assay. In states such as Florida and California, which historically have relied heavily upon the use of sentinel chickens for surveillance, an HI or EIA is used as a screening assay and FA or PRNT for confirmation. Virus infection rates in mosquitoes determined with in situ EIA methods (Graham *et al.*, 1986) also will be complicated by the rapid growth of WN compared to SLE in tissue culture and the cross-reactivity of the detector antibody. It may be necessary to add separate WN culture plates to surveillance systems, increasing the cost of testing large numbers of pools. As a result of these complications, diagnostics and surveillance may have to focus on the detection of virus by RT-PCR or plaque assay. Certainly the detection of infections in mosquitoes and dead birds will come to the forefront in enzootic surveillance.

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ADVANCES IN VIRUS RESEARCH, VOL 61

WEST NILE VIRUS: EPIDEMIOLOGY AND ECOLOGY IN NORTH AMERICA

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I. Introduction

The emergence of West Nile virus (WNV) in eastern North America in 1999 was a major event in modern arbovirology, not because of its disease impact or the potential threat it represented, but because it alerted the world that pathogens may turn up anywhere at any time. Bioterrorism awareness in the United States was increasing and an expected spin-off was preparedness for emerging infectious diseases. However, New York City, the epicenter of the 1999 outbreak, had no capacity for surveillance and control of arboviral diseases. Thus, WNV exposed a great weakness in the U.S. public health system. As a result of the outbreak, and the subsequent spread of WNV across the continent, a surge of resources became available to retool the

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public health system for arbovirus surveillance, prevention, and control. These resources have been used to initiate WNV surveillance in almost every state and province of the United States and Canada, and to initiate short- and long-term research projects aimed at understanding the biology of WNV in North America. This article will summarize the background information on this subject, and review the progress made in understanding WNV epidemiology and ecology in the New World.

II. History

WNV was first recognized in 1937 after it was isolated from blood of a febrile woman in the West Nile District of Uganda (Smithburn *et al.*, 1940). It became known as the etiologic agent of West Nile fever and was occasionally isolated from febrile children in North Africa and the Middle East beginning in the 1950s (Hayes, 2001). The occurrence of a dozen WNV encephalitis cases among elderly victims in Israel in 1957 was the first indication that WNV could cause serious central nervous system infections (Spigland *et al.*, 1958). Equine encephalitis caused by WNV was first noted in the early 1960s, in Egypt and France (Murgue *et al.*, 2001b; Schmidt and El Mansoury, 1963). In 1974, the largest known outbreak of WNV disease caused approximately 10,000 human fever cases in South Africa (Jupp, 2001; McIntosh *et al.*, 1976). In 1996, WNV emerged as a major cause of arboviral encephalitis in Romania, where an outbreak led to 393 recognized human cases of encephalitis, with 16 deaths (Tsai *et al.*, 1998). After 1996, outbreaks of West Nile viral encephalitis in people and horses were reported with increasing frequency in the Mediterranean Basin (Hubalek and Halouzka, 1999; Triki *et al.*, 2001), Russia (Platonov *et al.*, 2001) and Australia (Brown *et al.*, 2002). In 1997, a new strain of WNV that kills young domestic geese (*Anser* spp.) was isolated in Israel (Malkinson and Banet, 2002). An identical strain emerged in New York City in 1999 (Roehrig *et al.*, 2002).

In North America, the New York 1999 (NY99) strain of WNV was first isolated from a dead American Crow* (Lanciotti *et al.*, 1999) and subsequently from carcasses of 22 other bird species collected between August and November 1999 (Anderson *et al.*, 1999; Eidson *et al.*, 2001; Steele *et al.*, 2000). Simultaneously, WNV-specific RNA

*Latin names are provided for most bird species in Table III; otherwise, they are provided in the text.

sequences were identified from brain specimens collected from autopsies of fatal human cases (Briese *et al.*, 1999; Lanciotti *et al.*, 1999). However, the initial human cases were identified on the basis of serologic tests, which indicated that the North American St. Louis encephalitis virus (SLEV) was the likely etiologic agent (Roehrig *et al.*, 2002). SLEV is closely related to WNV, and these viruses cross-react in serologic tests. Subsequent to the identification of WNV, serologic results were reevaluated to include WNV in the testing panel, and stronger serologic reactions to WNV were observed in the patients' sera than to SLEV (Martin *et al.*, 2002).

After the initial North American outbreak in 1999, WNV overwintered in New York, with mid-winter infections discovered in hibernating mosquitoes (Nasci *et al.*, 2001b) and a fresh carcass of a Red-tailed Hawk (Garmendia *et al.*, 2000). After 1999, WNV continued to cause sporadic equine and human disease in the United States (CDC, 2002a; Marfin *et al.*, 2001), reaching Canada in 2001. In 2002, the largest outbreak of WNV encephalitis ever recorded occurred in the United States, with numerous epicenters spread across the nation's mid-section, and virus activity occurring coast-to-coast, breaching both the Canadian (Pepperell *et al.*, 2003) and Mexican borders (Blitvich *et al.*, 2003; Loroño-Pino *et al.*, 2003).

III. Clinical Description

A. *Human*

WNV infection in humans causes a spectrum of manifestations from subclinical infection to death (Petersen and Marfin, 2002). Most infections are subclinical but occasionally clinical manifestations will develop 2–21 days after infection. Cases lacking neurologic manifestations generally do not require hospitalization, and are termed "West Nile fever" (WNF). Neurologic cases usually involve meningoencephalitis, and have been termed "West Nile meningoencephalitis" (WNME). Asnis *et al.* (2000) published observations from a set of eight WNME patients evaluated in the 1999 New York City outbreak. The clinical picture in this group was similar to findings in Europe (Ceausu *et al.*, 1997) and the Middle East (Chowers *et al.*, 2001). The most common symptoms of cases requiring hospitalization were fever, gastrointestinal complaints, and change in mental status. Half the patients reported headache or severe muscle weakness. Two larger studies evaluated clinical characteristics of 59 and 19 hospitalized

patients, respectively (Nash *et al.*, 2001; Weiss *et al.*, 2001). No one manifestation was common to all cases, but general malaise, fatigue and flu-like gastrointestinal symptoms were common (Table I). Rare characteristics affecting less than 10% of the patients are not shown in Table I, and included tremors, shortness of breath, slurred speech, abdominal pain, focal sensory changes, pharyngitis, conjunctivitis, seizures, and lymphadenopathy. Hypertension, diabetes mellitus, and coronary artery disease were the most common underlying medical problems. Immunosuppression had been documented in 14% of the 1999 patients.

Two characteristics common in previous outbreaks of West Nile fever have been a generalized roseolar or macropapular rash and lymph-adeopathy. However, these signs have occurred in proportionally fewer cases in North America (Campbell *et al.*, 2002).

Unexpected findings in North America have included a poliomyelitis-like flaccid paralysis (Asnis *et al.*, 2000; Solomon and Ravi, 2003), Guillain-Barré syndrome (Ahmed *et al.*, 2000), profound muscle weakness (Sampson and Armbrustmacher, 2001), and eye abnormalities including uveitis, vitritis, and chorioretinitis (Bains *et al.*, 2003). Neurologic sequelae caused complications in 31% of a cohort of elderly WNME survivors (Berner *et al.*, 2002), but may affect as many as 50% (Petersen and Marfin, 2002; Weiss *et al.*, 2001), and often require long-term rehabilitation. Further studies are needed to characterize these sequelae.

Recent pathologic studies in a small number of fatal human cases (Sampson *et al.*, 2000; Shieh *et al.*, 2000) have confirmed similar findings as with Japanese encephalitis (reviewed in Solomon and Vaughn, 2002), including formation of microglial nodules and perivascular cuffing in the brain parenchyma. Lesions have been observed in the brainstem, spinal cord, thalamus, cortex, and cerebellum.

B. Equine

WNV infection in horses and other domestic equids ranges from asymptomatic to fatal encephalitis. A higher proportion of infected horses develop encephalitis compared with humans. Experimental studies suggest that about 10% of infected horses develop clinical illness (Bunning *et al.*, 2002). In naturally infected horses, WNV infection typically causes attitudinal changes (somnolence, listlessness, apprehension, depression, or hyperexcitability) and neurologic signs including muscle fasciculations and limb paresis or paralysis (Table II).

TABLE I
CLINICAL CHARACTERISTICS OF 78 PATIENTS HOSPITALIZED WITH WEST NILE VIRUS INFECTION IN 1999–2000

Signs and Symptoms	No. Patients	%
Fever	70	90
Weakness	41	53
Headache	39	50
Nausea	39	50
Vomiting	38	49
Altered mental status	38	49
Diarrhea	19	24
Stiff neck	17	22
Myalgia	16	21
Rash	14	18
Cough	14	18
Photophobia	14	18
Arthralgia	9	12

From Nash *et al.*, 2001; Weiss *et al.*, 2001.

TABLE II
CLINICAL SIGNS IN HORSES WITH WEST NILE ENCEPHALITIS

Sign	%
Ataxia	85
Weakness of limbs	48
Recumbency	45
Muscle fasciculation	40
Fever	23
Paralyzed or drooping lip	18
Tritching face or muzzle	13
Teeth grinding	7
Blindness	5

From Ostlund *et al.*, 2001.

Pathologic findings of horses infected in North America have not yet been reported. They are presumably similar to pathology described for the 1998 equine outbreak in Italy, in which spinal cord was the most affected tissue (Cantile *et al.*, 2000). Histologic lesions were observed

in the brain stem and gray matter of the spinal cord in a fatal case of equine WNV in Israel in 2000 (Steinman *et al*., 2002).

C. Avian

WNV-infected birds also suffer a spectrum of clinical outcomes ranging from no disease to death. Mortality attributable to WNV infection in North America has been reported in 198 species of birds through 2002 (Table III). Some species of birds, especially corvids* are highly susceptible to fatal outcome (Komar *et al*., 2003a; McLean *et al*., 2002). General signs of infection include lethargy, recumbency, and in some cases, hemorrhage (Komar *et al*., 2003a). Swayne *et al*. (2001) documented abnormal posture in a domestic gosling (*Anser anser domesticus*). Because of the high rates of natural infection in birds during epizootics (see Section VI,C), disease in seropositive birds may be difficult to attribute to WNV infection. This is particularly a problem for captive birds such as those in zoos and wildlife rehabilitation centers, where veterinary care favors survival of birds with chronic conditions. Many of these birds are seropositive for WNV, but the etiology of their clinical signs (such as blindness in great horned owls) remains unknown.

In North America, gross and histopathologic studies have described the pathogenesis of natural, acute fatal WNV infection in birds for 14 species, representing eight orders (Steele *et al*., 2000) and experimental infection in domestic geese (Swayne *et al*., 2001). Brain hemorrhage, splenomegaly, meningoencephalitis, and myocarditis were the prominent findings on gross examination. Numerous cell types were damaged, in various tissues. Purkinje cells were particulary targeted except in corvids. The cause of death in most of these birds is probably multiple organ failure.

D. Other Vertebrates

Little is known of the clinical manifestation of WNV in other vertebrates, such as reptiles and amphibians and other mammals. In North America, captive alligators have died from WNV infection (Miller *et al*., 2003), and fatal infections have been informally reported through 2002 in approximately 20 species of mammals in addition to horses and people (see Section VI,B).

*Members of the family *Corvidae*, Order Passeriformes.

TABLE III

List of 198 Bird Species Fatally Affected by West Nile Virus in North America[a,b,c]

Common Name	Latin Name	Family	Order	Status[d]
Elegant crested tinamou	*Eudromia elegans*	Tinamidae	Tinamiformes	Exotic[e]
Emu	*Dromaius novaehollandiae*	Dromaiidae	Casuariiformes	Exotic[e]
Common loon	*Gavia immer*	Gaviidae	Gaviiformes	Native
Pied-billed grebe	*Podilymbus podiceps*	Podicipedidae	Podicipediformes	Native
Humboldt penguin	*Spheniscus Humboldti*	Spheniscadae	Sphenisciformes	Exotic[e]
Black-footed penguin	*Spheniscus demersus*	Spheniscadae	Sphenisciformes	Exotic[e]
American white pelican	*Pelecanus erythrorhynchos*	Pelecanidae	Pelecaniformes	Native
Double-crested cormorant	*Phalacrocorax auritus*	Phalacrocoracidae	Pelecaniformes	Native
Guanay cormorant	*Phalacrocorax bougainvillei*	Phalacrocoracidae	Pelecaniformes	Exotic[e]
Least bittern	*Ixobrychus exilis*	Ardeidae	Ciconiiformes	Native
Great blue heron	*Ardea herodias*	Ardeidae	Ciconiiformes	Native
Great egret	*Ardea alba*	Ardeidae	Ciconiiformes	Native
Green heron	*Butorides virescens*	Ardeidae	Ciconiiformes	Native
Black-crowned night heron	*Nycticorax nycticorax*	Ardeidae	Ciconiiformes	Native
Yellow-crowned night heron	*Nyctanassa violacea*	Ardeidae	Ciconiiformes	Native
Scarlet ibis	*Eudocimus ruber*	Threskiornithidae	Ciconiiformes	Exotic[e]
Black vulture	*Coragyps atratus*	Cathartidae	Ciconiiformes	Native
Turkey vulture	*Cathartes aura*	Cathartidae	Ciconiiformes	Native
Chilean flamingo	*Phoenicopterus chilensis*	Phoenicopteridae	Phoenicopteriformes	Exotic[e]
Greater flamingo	*Phoenicopterus ruber*	Phoenicopteridae	Phoenicopteriformes	Exotic[e]
Canada goose	*Branta canadensis*	Anatidae	Anseriformes	Native
Hawaiian goose	*Branta sandvicensis*	Anatidae	Anseriformes	Exotic[e]

(continues)

TABLE III (*continued*)

Common Name	Latin Name	Family	Order	Status[d]
Red-breasted goose	*Branta ruficollis*	Anatidae	Anseriformes	Exotic[e]
Emperor goose	*Chen canagica*	Anatidae	Anseriformes	Native[e]
Mute swan	*Cygnus olor*	Anatidae	Anseriformes	Introduced
Tundra swan	*Cygnus columbianus*	Anatidae	Anseriformes	Native[e]
Wood duck	*Aix sponsa*	Anatidae	Anseriformes	Native
Bronze-winged duck	*Anas specularis*	Anatidae	Anseriformes	Exotic[e]
Eurasian wigeon	*Anas penelope*	Anatidae	Anseriformes	Native[e]
Mallard	*Anas platyrhynchos*	Anatidae	Anseriformes	Native
Cinnamon teal	*Anas cyanoptera*	Anatidae	Anseriformes	Native[e]
Yellow-billed duck	*Anas undulata*	Anatidae	Anseriformes	Exotic[e]
Puna teal	*Anas puna*	Anatidae	Anseriformes	Exotic[e]
Canvasback	*Aythya valisineria*	Anatidae	Anseriformes	Native
Greater scaup	*Aythya marila*	Anatidae	Anseriformes	Native[e]
Lesser scaup	*Aythya affinis*	Anatidae	Anseriformes	Native[e]
Bufflehead	*Bucephala albeola*	Anatidae	Anseriformes	Native[e]
Common goldeneye	*Bucephala clangula*	Anatidae	Anseriformes	Native[e]
Smew	*Mergellus albellus*	Anatidae	Anseriformes	Exotic[e]
Common merganser	*Mergus merganser*	Anatidae	Anseriformes	Native[e]
Ruddy duck	*Oxyura jamaicensis*	Anatidae	Anseriformes	Native
Osprey	*Pandion haliaetus*	Accipitridae	Falconiformes	Native
Swallow-tailed kite	*Elanoides forficatus*	Accipitridae	Falconiformes	Native
Mississippi kite	*Ictinia mississippiensis*	Accipitridae	Falconiformes	Native
Bald eagle	*Haliaeetus leucocephalus*	Accipitridae	Falconiformes	Native[e]

Northern harrier	*Circus cyaneus*	Accipitridae	Falconiformes	Native
Sharp-shinned hawk	*Accipiter striatus*	Accipitridae	Falconiformes	Native
Cooper's hawk	*Accipiter cooperii*	Accipitridae	Falconiformes	Native
Northern goshawk	*Accipiter gentilis*	Accipitridae	Falconiformes	Native
Harris' hawk	*Parabuteo unicinctus*	Accipitridae	Falconiformes	Native[e]
Red-shouldered hawk	*Buteo lineatus*	Accipitridae	Falconiformes	Native
Broad-winged hawk	*Buteo platypterus*	Accipitridae	Falconiformes	Native
Swainson's hawk	*Buteo swainsoni*	Accipitridae	Falconiformes	Native
Red-tailed hawk	*Buteo jamaicensis*	Accipitridae	Falconiformes	Native
Rough-legged hawk	*Buteo lagopus*	Accipitridae	Falconiformes	Native[e]
Golden eagle	*Aquila chrysaetos*	Accipitridae	Falconiformes	Native[e]
Wedge-tailed eagle	*Aquila audax*	Accipitridae	Falconiformes	Exotic[e]
American kestrel	*Falco sparverius*	Falconidae	Falconiformes	Native
Merlin	*Falco columbarius*	Falconidae	Falconiformes	Native
Prairie falcon	*Falco mexicanus*	Falconidae	Falconiformes	Native[e]
Peregrine falcon	*Falco peregrinus*	Falconidae	Falconiformes	Native
Domestic chicken	*Gallus gallus*	Phasianidae	Galliformes	Exotic[e]
Ring-necked pheasant	*Phasianus colchicus*	Phasianidae	Galliformes	Introduced
Impeyan pheasant	*Lophophorus impeyanus*	Phasianidae	Galliformes	Exotic[e]
Monal pheasant	*Lophophorus ihuysii*	Phasianidae	Galliformes	Exotic[e]
Common peafowl	*Pavo cristatus*	Phasianidae	Galliformes	Exotic[e]
Blythe's tragopan	*Tragopan blythi*	Phasianidae	Galliformes	Exotic[e]
Satyr tragopan	*Tragopan satyr*	Phasianidae	Galliformes	Exotic[e]
Ruffed grouse	*Bonasa umbellus*	Phasianidae	Galliformes	Native
Wild turkey	*Meleagris gallopavo*	Phasianidae	Galliformes	Native
Northern bobwhite	*Colinus virginianus*	Odontophoridae	Galliformes	Native

(*continues*)

TABLE III (*continued*)

Common Name	Latin Name	Family	Order	Status[d]
Virginia rail	*Rallus limicola*	Rallidae	Gruiformes	Native
Sandhill crane	*Grus canadensis*	Gruidae	Gruiformes	Native[e]
Killdeer	*Charadrius vociferus*	Charadriidae	Charadriiformes	Native
Ruddy turnstone	*Arenaria interpres*	Scolopacidae	Charadriiformes	Native
Laughing gull	*Larus atricilla*	Laridae	Charadriiformes	Native
Ring-billed gull	*Larus delawarensis*	Laridae	Charadriiformes	Native
Herring gull	*Larus argentatus*	Laridae	Charadriiformes	Native
Great black-backed gull	*Larus marinus*	Laridae	Charadriiformes	Native
Inca tern	*Larosterna inca*	Laridae	Charadriiformes	Exotic[e]
Black skimmer	*Rhynchops niger*	Laridae	Charadriiformes	Native
Rock dove	*Columba livia*	Columbidae	Columbiformes	Introduced
White-crowned pigeon	*Columba leucocephala*	Columbidae	Columbiformes	Native
Eurasian collared-dove	*Streptopelia decaocto*	Columbidae	Columbiformes	Introduced
White-winged dove	*Zenaida asiatica*	Columbidae	Columbiformes	Native
Mourning dove	*Zenaida macroura*	Columbidae	Columbiformes	Native
Common ground-dove	*Columbina passerina*	Columbidae	Columbiformes	Native
Budgerigar	*Melopsittacus undulatus*	Psittacidae	Psittaciformes	Introduced[e]
Pacific parrotlet	*Forpus coelestis*	Psittacidae	Psittaciformes	Exotic[e]
Macaw	*Ara* species	Psittacidae	Psittaciformes	Exotic[e]
Red-crowned parrot	*Amazona viridigenalis*	Psittacidae	Psittaciformes	Exotic[e]
Thick-billed parrot	*Rhynchopsitta pachyrhyncha*	Psittacidae	Psittaciformes	Exotic[e]
Rainbow lorikeet	*Trichoglossus haematodus*	Psittacidae	Psittaciformes	Exotic[e]
Violet-necked lorikeet	*Eos beckstein*	Psittacidae	Psittaciformes	Exotic[e]

Blue-streaked lory	*Eos reticulate*	Psittacidae	Psittaciformes	Exotic[e]
Red lory	*Eos bornea*	Psittacidae	Psittaciformes	Exotic[e]
Dusky lory	*Pseudeos fuscata*	Psittacidae	Psittaciformes	Exotic[e]
Black-capped lory	*Lorius lory*	Psittacidae	Psittaciformes	Exotic[e]
Crimson rosella	*Platycercus elegans*	Psittacidae	Psittaciformes	Exotic[e]
Cockatoo	*Cacatua* species	Cacatuidae	Psittaciformes	Exotic[e]
Cockatiel	*Nymphicus hollandicus*	Cacatuidae	Psittaciformes	Exotic[e]
Yellow-billed cuckoo	*Coccyzus americanus*	Cuculidae	Cuculiformes	Native
Barn owl	*Tyto alba*	Tytonidae	Strigiformes	Native
Eastern screech-owl	*Otus asio*	Strigidae	Strigiformes	Native
Great horned owl	*Bubo virginianus*	Strigidae	Strigiformes	Native
Snowy owl	*Nyctea scandiaca*	Strigidae	Strigiformes	Native[e]
Northern hawk owl	*Surnia ulula*	Strigidae	Strigiformes	Native[e]
Spotted owl	*Strix occidentalis*	Strigidae	Strigiformes	Native[e]
Barred owl	*Strix varia*	Strigidae	Strigiformes	Native
Great gray owl	*Strix nebulosa*	Strigidae	Strigiformes	Native[e]
Long-eared owl	*Asio otus*	Strigidae	Strigiformes	Native
Tawny owl	*Strix aluco*	Strigidae	Strigiformes	Exotic[e]
Short-eared owl	*Asio flammeus*	Strigidae	Strigiformes	Native
Boreal owl	*Aegolius funereus*	Strigidae	Strigiformes	Native[e]
Northern saw-whet owl	*Aegolius acadicus*	Strigidae	Strigiformes	Native
Common nighthawk	*Chordeiles minor*	Caprimulgidae	Caprimulgiformes	Native
Chimney swift	*Chaetura pelagica*	Apodidae	Apodiformes	Native
Ruby-throated hummingbird	*Archilochus colubris*	Trochilidae	Apodiformes	Native
Belted kingfisher	*Ceryle alcyon*	Alcedinidae	Coraciiformes	Native
Micronesian kingfisher	*Halcyon cinnamomima*	Alcedinidae	Coraciiformes	Exotic[e]

(*continues*)

TABLE III (*continued*)

Common Name	Latin Name	Family	Order	Status[d]
Abyssinian ground-hornbill	*Bucorvus abyssinicus*	Bucorvidae	Coraciiformes	Exotic[e]
Red-headed woodpecker	*Melanerpes erythrocephalus*	Picidae	Piciformes	Native
Yellow-bellied sapsucker	*Sphyrapicus varius*	Picidae	Piciformes	Native
Downy woodpecker	*Picoides pubescens*	Picidae	Piciformes	Native
Traill's flycatcher	*Empidonax trailliialnorum*	Tyrannidae	Passeriformes	Native
Eastern phoebe	*Sayornis phoebe*	Tyrannidae	Passeriformes	Native
Eastern kingbird	*Tyrannus tyrannus*	Tyrannidae	Passeriformes	Native
Scissor-tailed flycatcher	*Tyrannus forficatus*	Tyrannidae	Passeriformes	Native
Loggerhead shrike	*Lanius ludovicianus*	Laniidae	Passeriformes	Native
Warbling vireo	*Vireo gilvus*	Vireonidae	Passeriformes	Native
Red-eyed vireo	*Vireo olivaceus*	Vireonidae	Passeriformes	Native
Black-whiskered vireo	*Vireo altiloquus*	Vireonidae	Passeriformes	Native
Eurasian jay	*Garrulus glandarius*	Corvidae	Passeriformes	Exotic[e]
Steller's jay	*Cyanocitta stelleri*	Corvidae	Passeriformes	Native
Blue jay	*Cyanocitta cristata*	Corvidae	Passeriformes	Native
Western scrub-jay	*Aphelocoma californica*	Corvidae	Passeriformes	Native
Clark's nutcracker	*Nucifraga columbiana*	Corvidae	Passeriformes	Native[e]
Black-billed magpie	*Pica hudsonia*	Corvidae	Passeriformes	Native
American crow	*Corvus brachyrhynchos*	Corvidae	Passeriformes	Native
Fish crow	*Corvus ossifragus*	Corvidae	Passeriformes	Native
Hooded crow	*Corvus corone*	Corvidae	Passeriformes	Exotic[e]
Common raven	*Corvus corax*	Corvidae	Passeriformes	Native
Purple martin	*Progne subis*	Hirundinidae	Passeriformes	Native

Barn swallow	*Hirundo rustica*	Hirundinidae	Passeriformes	Native
Varied tit	*Parus varius*	Paridae	Passeriformes	Exotic[e]
Carolina chickadee	*Poecile carolinensis*	Paridae	Passeriformes	Native
Black-capped chickadee	*Poecile atricapillus*	Paridae	Passeriformes	Native
Tufted titmouse	*Parus bicolor*	Paridae	Passeriformes	Native
White-breasted nuthatch	*Sitta carolinensis*	Sittadae	Passeriformes	Native
Carolina wren	*Thryothaurus ludovicianus*	Troglodytidae	Passeriformes	Native
Winter wren	*Troglodytes troglodytes*	Troglodytidae	Passeriformes	Native
Eastern bluebird	*Sialia sialis*	Turdidae	Passeriformes	Native
Veery	*Catharus fuscescens*	Turdidae	Passeriformes	Native
Gray-cheeked thrush	*Catharus minimus*	Turdidae	Passeriformes	Native
Swainson's thrush	*Catharus ustulatus*	Turdidae	Passeriformes	Native
Hermit thrush	*Catharus guttatus*	Turdidae	Passeriformes	Native
Wood thrush	*Hylocichla mustelina*	Turdidae	Passeriformes	Native
American robin	*Turdus migratorius*	Turdidae	Passeriformes	Native
Gray catbird	*Dumetella carolinensis*	Mimidae	Passeriformes	Native
Northern mockingbird	*Mimus polyglottos*	Mimidae	Passeriformes	Native
Brown thrasher	*Toxostoma rufum*	Mimidae	Passeriformes	Native
European starling	*Sturnus vulgaris*	Sturnidae	Passeriformes	Introduced
Cedar waxwing	*Bombycilla cedrorum*	Bombycillidae	Passeriformes	Native
Nashville warbler	*Vermivora ruficapilla*	Parulidae	Passeriformes	Native
Northern parula	*Parula americana*	Parulidae	Passeriformes	Native
Yellow warbler	*Dendroica petechia*	Parulidae	Passeriformes	Native
Black-throated blue warbler	*Dendroica caerulescens*	Parulidae	Passeriformes	Native
Yellow-rumped warbler	*Dendroica coronata*	Parulidae	Passeriformes	Native
Blackpoll warbler	*Dendroica striata*	Parulidae	Passeriformes	Native

(*continues*)

TABLE III (*continued*)

Common Name	Latin Name	Family	Order	Status[d]
Ovenbird	*Seiurus aurocapillus*	Parulidae	Passeriformes	Native
Northern waterthrush	*Seiurus noveboracensis*	Parulidae	Passeriformes	Native
Kentucky warbler	*Oporornis formosus*	Parulidae	Passeriformes	Native
Common yellowthroat	*Geothlypis trichas*	Parulidae	Passeriformes	Native
Hooded warbler	*Wilsonia citrina*	Parulidae	Passeriformes	Native
Canada warbler	*Wilsonia canadensis*	Parulidae	Passeriformes	Native
American goldfinch	*Carduelis tristis*	Fringillidae	Passeriformes	Native
Eastern towhee	*Pipilo erythrophthalmus*	Emberizidae	Passeriformes	Native
Field sparrow	*Spizella pusilla*	Emberizidae	Passeriformes	Native
Savannah sparrow	*Passerculus sandwichensis*	Emberizidae	Passeriformes	Native
Fox sparrow	*Passerella iliaca*	Emberizidae	Passeriformes	Native
Song sparrow	*Melospiza melodia*	Emberizidae	Passeriformes	Native
Northern cardinal	*Cardinalis cardinalis*	Cardinalidae	Passeriformes	Native
Rose-breasted grosbeak	*Pheucticus ludovicianus*	Cardinalidae	Passeriformes	Native
Dickcissel	*Spiza americana*	Cardinalidae	Passeriformes	Native
Red-winged blackbird	*Agelaius phoeniceus*	Icteridae	Passeriformes	Native
Rusty blackbird	*Euphagus carolinus*	Icteridae	Passeriformes	Native
Brewer's blackbird	*Euphagus cyanocephalus*	Icteridae	Passeriformes	Native
Common grackle	*Quiscalus quiscula*	Icteridae	Passeriformes	Native
Boat-tailed grackle	*Quiscalus major*	Icteridae	Passeriformes	Native
Great-tailed grackle	*Quiscalus mexicanus*	Icteridae	Passeriformes	Native
Brown-headed cowbird	*Molothrus ater*	Icteridae	Passeriformes	Native
Baltimore oriole	*Icterus galbula*	Icteridae	Passeriformes	Native

Purple finch	*Carpodacus purpureus*	Fringillidae	Passeriformes	Native
House finch	*Carpodacus mexicanus*	Fringillidae	Passeriformes	Native
European goldfinch	*Carduelis carduelis*	Fringillidae	Passeriformes	Exotic[e]
Evening grosbeak	*Coccothraustes vespertinus*	Fringillidae	Passeriformes	Native
House sparrow	*Passer domesticus*	Passeridae	Passeriformes	Introduced
Zebra finch	*Taeniopygia guttata*	Estrildidae	Passeriformes	Exotic[e]

[a] Compiled through 2002 from reports to Centers for Disease Control and Prevention's ArboNET surveillance databank (CDC, unpublished data), CDC's WNV-zoo surveillance program (Dominic Travis, Amy Glaser, personal communication), U.S.G.S. National Wildlife Health Center (Emi K. Saito, personal communication), Canadian Wildlife Service (Ian Barker, personal communication), and peer-reviewed publications.

[b] Classification follows the A.O.U. Check-list of North American Birds, Seventh Edition (AOU, 2002).

[c] The correct identification of these species is not guaranteed.

[d] "Native" refers to species naturally occurring in North America; "Introduced" refers to non-native (exotic) species that have established free-ranging populations; "Exotic" indicates non-native species, without established free-ranging populations.

[e] Bird(s) died in captivity only.

IV. Geographic Distribution

The geographic distribution of WNV is known from human and equine outbreaks, avian epizootics (particularly in North America) and serosurveys of vertebrate hosts (Hayes, 1989; Hubalek and Halouzka, 1999; Komar, 2000). In Africa, WNV is known from most countries where arbovirus studies have been conducted, from South Africa and Madagascar in the extreme south to Morocco, Algeria, Tunisia, and Egypt in the north. Its range extends from Africa eastward through the Middle East into south Asia, where it is known from Pakistan and India. It extends northward into southern Russia, and westward through southern Europe. A variant of WN virus, Kunjin virus, is present in Australia and contiguous regions of Southeast Asia (Hall *et al.*, 2002; Scherret *et al.*, 2001).

In North America through the end of 2002, WNV had spread to every continental U.S. state except Oregon, Utah, Nevada, and Arizona (CDC, 2002k); the Canadian provinces of Saskatchewan, Manitoba, Ontario, Quebec, and Nova Scotia (P. Buck, personal communication); the Mexican states of Coahuila (Blitvich *et al.*, 2003) and Yucatan (Loroño-Pino *et al.*, 2003); the Cayman Islands (CDC, 2002a), Jamaica (DuPuis *et al.*, 2003), and the Dominican Republic (Komar *et al.*, 2003b) in the Caribbean Basin (Fig. 1).

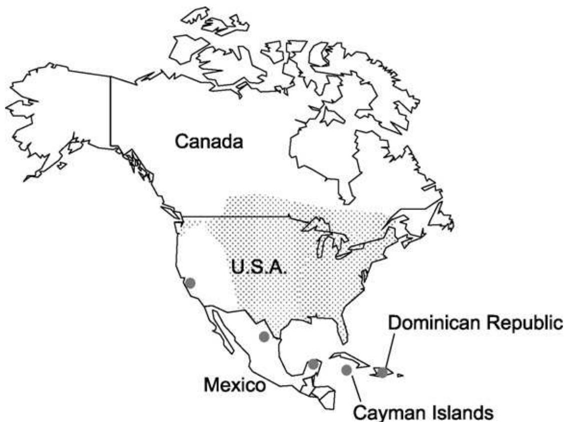

Fig 1. Distribution of West Nile virus in North America through 2002. The single points on southern California and the Cayman Islands represent single human cases, whereas the points in Mexico, Jamaica and the Dominican Republic represent serologic evidence for local transmission in horses (Mexico) and birds (Caribbean nations).

V. Molecular Epidemiology

WNV is a *Flavivirus* (family *Flaviviridae*). Its structure and size are similar to other flaviviruses, including the prototype, yellow fever virus. A large body of knowledge of the molecular biology of WNV has recently been reviewed (Brinton, 2002). Numerous strains of WNV have been isolated, separated by time and space, since 1937. Phylogenetically these strains make up a grouping of closely related viruses (Fig. 2). At least two separate genetic lineages of WNV have been described (Berthet *et al.*, 1997; Burt *et al.*, 2002; Lanciotti *et al.*, 1999, 2002; Scherret *et al.*, 2001). Lanciotti *et al.* place all of the European, Middle Eastern, South Asian, Australian (Kunjin virus), and North American strains in Lineage 1. This lineage includes the strains that have caused encephalitis outbreaks in humans and horses. It also includes some African strains. Lineage 2 includes southern African strains, including some from central Africa and the Ugandan prototype strain isolated in 1937. Although Lineage 2 viruses have not been associated with outbreaks of severe disease, one South African strain was responsible for the largest WNF outbreak recorded, with over 10,000 mild fever cases in 1974. However, these strains have caused only isolated cases of human encephalitis and hepatitis, canine and equine

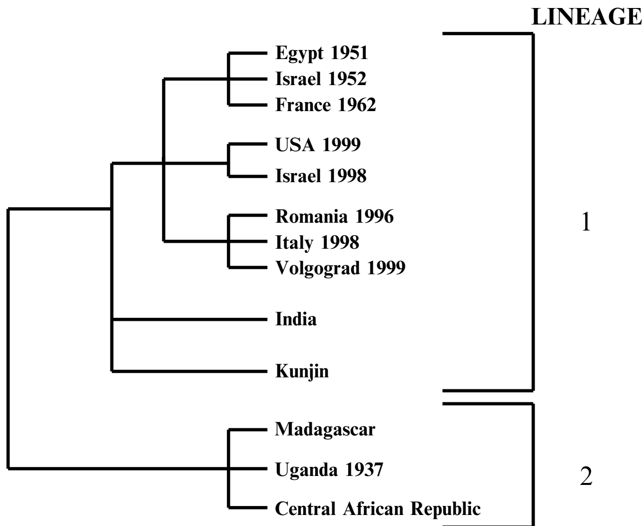

FIG 2. Simplified representation of a phylogenetic tree showing the genetic relatedness of different strains of West Nile virus, including separation into two distinct lineages.

encephalitis, and a fatality in an ostrich (*Struthio camelus*) chick (Burt *et al.*, 2002). Scherret *et al.* (2001) suggest that four or five separate WNV subgroups may be recognized. In this phylogeny, the south Asian, Malaysian, and Australian strains form three additional subgroups in addition to the Lineage 1 and 2 groups.

WNV is most closely related to other flaviviruses of the Japanese encephalitis antigenic complex, which includes Japanese encephalitis virus (JEV) in Asia; Murray Valley encephalitis (MVEV) and Alfuy viruses in Australia; Koutango and Usutu viruses in Africa; and SLEV, Rocio, Ilheus, and Cacipacore viruses in the Americas (Calisher *et al.*, 1989). Interestingly, a phylogenetic analysis determined that SLEV, although it cross-reacts with JEV serocomplex virus such as WNV, in fact falls within a separate clade that includes members of the Ntaya serocomplex (Kuno *et al.*, 1998). Serologic cross-reactions between WNV and SLEV occur at relatively low levels (Johnson *et al.*, 2003; Komar *et al.*, 2001a; Martin *et al.*, 2002). Of WNV's close relatives, a number cause encephalitis in humans (JEV, SLEV, MVEV, Rocio), and Usutu virus has been recently discovered as the etiologic agent of fatal infections of European blackbirds (*Turdus merula*) and other birds in Austria (Weissenbock *et al.*, 2002).

VI. Epidemiology and Epizootiology

A. *Disease Incidence in Humans*

In North America, human cases of WNME were first detected in New York City in August 1999 (Nash *et al.*, 2001), and continued to occur at low levels in 2000 and 2001 in a consistently growing geographic area. In 2002, case numbers increased exponentially (Table IV). Case-fatality rates (CFR) have varied over time and space, depending mainly on the local definition of a case and upon the intensity of surveillance for mild cases. Overall in the United States since 1999, there have been 217 deaths reported (through November 30, 2002) and 3536 cases, indicating a cumulative CFR of 6.1%. If nonneurologic cases (i.e., West Nile fever) are excluded, the CFR would be higher (8.5% in 2002; CDC, 2002k).

Clusters of human cases suggestive of focal outbreaks occurred in New York in 1999 (Nash *et al.*, 2001) and 2000 (Weiss *et al.*, 2001), and in the following states (greater than 100 cases) in 2002: Illinois (492 cases), Michigan (437), Ohio (277), Louisiana (202), and Texas (164) (CDC, 2002k).

TABLE IV
REPORTED NUMBER OF HUMAN AND EQUINE WEST NILE VIRUS DISEASE CASES AND CASE-FATALITY RATES, USA, 1999–2002[a]

Year	Human			Equine		
	Cases	Deaths	CFR	Cases	Deaths	CFR
1999	62	7	11.3%	20	9	40.0%
2000	21	2	9.5%	60	23	38.3%
2001	64	9	14.1%	733	N.R.	N.R.
2002[b]	3389	199	5.9%	9144	N.R.	N.R.

N.R., not reported.
CFR, Case-fatality rate.
[a] Canadian cases not included in this table. In 2002, Health Canada reported 390 human cases.
[b] Data for 2002 is incomplete, and includes data reported to CDC ArboNET through November 30, 2002 (Chow *et al*., 2002).

B. Disease Incidence in Other Vertebrates

Significant natural morbidity in non-human mammals has been reported only in equids, including horses, donkeys, and mules. Recent equine epizootics have been described in Morocco in 1996 (Tber, 1996), Italy in 1998 (Autorino *et al*., 2002), Israel in 2000 (Steinman *et al*., 2002), and France in 2000 (Murgue *et al*., 2001a). In North America, numbers of affected equids has increased annually since 1999 (Table IV). The initial equine outbreak was clustered on Long Island, NY, about 50 miles east of New York City, in September-October 1999 (Trock *et al*., 2001). The subsequent increase in cases reflects the geographic spread of WNV and the increase in the equine population at risk (CDC, 2002a, 2002k; Ostlund *et al*., 2001). The exponential increases in 2001 and 2002 probably also reflect spread of WNV into regions in which *Culex* vectors feed more frequently on horses, such as *Cx. quinquefasciatus* in the Southeast and *Cx. tarsalis* in the Great Plains states.

Whereas most other mammals appear to be susceptible to infection with WNV, few become ill or die. In North America, small numbers of disease cases and deaths attributed to WNV have occurred in squirrels (*Sciurus carolinensis* and *S. niger*) (CDC, 2002k; Heinz-Taheny *et al*., 2004; Marfin *et al*., 2001), an eastern chipmunk (*Tamias striatus*), a big brown bat (*Eptesicus fuscus*), a little brown bat (*Myotis lucifugas*), a striped skunk (*Mephitis mephitis*), a domestic rabbit (*Oryctologus cuniculus*) (Marfin *et al*., 2001), and a domestic cat (*Felis cattus*) (Komar,

2000). Three dogs were reported in 2002 (CDC, 2002k). Before the North American outbreak, the only previous report of WNV illness in mammals other than people and horses had described WNV infection in a dog from Botswana (Burt *et al.*, 2002; Simpson and Kuebart, 1979).

In 1997–2000, significant avian mortality was observed in Israel, with outbreaks in young domestic geese, as well as some migrating white storks (*Ciconia ciconia*), captive white-eyed gulls (*Larus leucophthalmus*), and a lappet-faced vulture (*Torgos traceliotus*) (Malkinson and Banet, 2002). Before 1997, the only report of natural WNV-associated morbidity in birds was a sick fledgling pigeon (a.k.a. rock dove) in Egypt in the early 1950s (Work *et al.*, 1953) and an isolate from a dead ostrich chick in South Africa in 1994 (Burt *et al.*, 2002). However, experimental infections in hooded crows (*Corvus corone sardonius*) and house sparrows resulted in 100% and 79% mortality, respectively (Work *et al.*, 1955). Experimental morbidity was also observed in black-tailed gulls (*Larus crassirostris*) and rooks (*Corvus frugilegus*) (reviewed in Hubalek and Halouzka, 1996), but not in 13 species of birds evaluated in South Africa (McIntosh *et al.*, 1969).

In North America, avian mortality has proven to be extensive. Natural fatal infections have been reported based on positive laboratory tests of over 28,000 carcasses between 1999 and 2002, representing 198 species of birds (CDC, unpublished data). Incidence of disease in birds, however, has not been well documented because most laboratory testing has been for public health surveillance purposes, and therefore effects of WNV disease on specific bird populations has generally not been reported. Anecdotal reports suggest that incidence in certain species has been extremely high, such as American crows, which may be experiencing 100% mortality in some regions. In Stillwater, Oklahoma, WNV was associated with 32% mortality in young crows in 2002 (Caffrey *et al.*, 2003). About half of the positive carcasses reported have indeed been identified as American crows. About half of the remainder is a closely related species within the Corvidae family, the blue jay. Specific mortality rates in some species of North American birds can be inferred from experimental infection studies. WNV-associated clinical signs were absent in 12 chickens inoculated by injection, although morbidity was suggested by histopathologic studies (Senne *et al.*, 2000, see Section III,B). No morbidity or mortality was observed in 21 chickens infected by mosquito bite (Langevin *et al.*, 2001). However, young chicks are known to succumb to WNV infection. Domestic geese (*Anser anser domesticus*) suffered 100% morbidity and 75% mortality in a study of four 2-week-old goslings (Swayne *et al.*, 2001).

TABLE V
MORTALITY OBSERVED IN EIGHT SPECIES OF NORTH AMERICAN BIRDS EXPOSED TO WEST NILE VIRUS BY MOSQUITO BITE

Species	No. Exposed	No. Fatal Infections (% of exposed)	Mean No. Days to Death (range)
Ring-billed gull	2	2 (100%)	9.0 (5–13)
Blue jay	4	3 (75%)	4.7 (4–5)
Black-billed magpie	3	3 (100%)	6.0 (6–6)
American crow	8	8 (100%)	5.1 (4–6)
Fish crow	9	5 (55%)	9.6 (6–13)
Common grackle	6	2 (33%)	4.5 (4–5)
House finch	2	2 (100%)	7.0 (6–8)
House sparrow	6	3 (50%)	4.7 (3–6)

From Komar *et al.*, 2003a.

Domestic turkeys were resistant to disease (Swayne *et al.*, 2000). An assortment of 25 species of birds representing 10 different orders and 17 families suffered varying degrees of mortality after infection by mosquito bite (Table V). Mortality was observed in eight species that developed high-titered viremias, in particular among the passerine birds (Komar *et al.*, 2003a). The majority of the birds in this study survived the acute phase of WNV infection, and developed neutralizing antibodies.

C. Seroprevalence

A seroprevalence study after the 1999 WNV epidemic in northeast Queens, New York City, found 2.6% of the resident human population to be positive. The number of cases reported from the same neighborhoods was used to estimate the ratio of cases to infections, which was 140:1. WNF symptoms were recalled by 21% of the seropositive respondents (Mostashari *et al.*, 2001). The ratio of cases to subclinical infections was similar to that determined for the Romania WNV outbreak of 1996 (Tsai *et al.*, 1998). Additional serosurveys of humans in the metropolitan NYC region after the 2000 epidemic in Staten Island confirmed the low infection rates in the general population (CDC, 2001; McCarthy *et al.*, 2001).

Seroprevalence studies in equines after WNV epizootics have also been reported. Apparently healthy stablemates of 1999 horse cases in

Long Island, New York, were seropositive at a rate of 29%, indicating a high rate of subclinical infections (Trock *et al.*, 2001). Infection of horses was also detected in Queens at the epicenter of the human outbreak, where one of 18 police horses was seropositive. In other boroughs of New York City, the infection rates of horses were even lower. The seroprevalence study of New York City horses in October 1999 was part of a study that included pet and stray dogs (and small number of pet cats as well) to evaluate whether infections in domestic mammals might be useful for surveillance purposes. No seropositive cats were detected, but nine of 80 dogs in Queens and the Bronx were seropositive (Komar *et al.*, 2001b). An equine serosurvey on Staten Island (the epicenter of a human outbreak) in 2000 detected seven seropositive horses of 91 surveyed (Trock *et al.*, 2001).

High seroprevalence has been found in birds in epizootic transmission foci. Most of these studies have been aimed at understanding the ecology of WNV proliferation, and are described in greater detail later (see Section VII,B). In summary, seroprevelances in resident birds was 50% and 23% in the epicenters of the 1999 and 2000 outbreaks, respectively (Komar *et al.*, 2001a, 2001c). In October 1999, the seroprevalence was evaluated for resident and migratory birds at the outskirts of New York City. Overall, 0.8% of 1018 birds sampled were seropositive for WNV (McLean *et al.*, 2002).

D. Risk Factors

Hayes (1989) reviewed risk factors in the Old World. The principal risk factor for infection was geographic location because WNV was noted to be active in certain well-defined locations within specific countries. It was also noted that advanced age was the principal risk factor for severe human disease. Han *et al.* (1999) found that time spent outdoors and in flooded basements were risk factors for infection during the 1996 outbreak in Bucharest, Romania. Bin *et al.* (2001) reported that close contact with sick geese was a risk factor for human infection in Israel in 1999, but not residence in areas along bird migration routes. The study of human risk factors for WNV infection and disease in North America is in its infancy.

In New York City in 1999, the initial series of eight patients had clustered residences within a 2-mile radius in Queens, and all had outdoor exposure (Asnis *et al.*, 2000). An analysis of the full series of 59 hospitalized cases determined that all had disease onsets between early August and late September (Nash *et al.*, 2001). Thus, risk is greatest in the New York City region during the third quarter of

the year coincident with the seasonal blood-feeding by mosquitoes. This seasonality of risk would be less restricted where mosquito blood-feeding is extended or occurs year-round.

The median age of human cases was 71 (range, 5–95) and the attack rate was 20 times greater in persons older than 50 years of age than in younger persons (Nash *et al.*, 2001). Age $\geq$75 years was a risk factor for death (relative risk 8.5), as was diabetes mellitus (age-adjusted relative risk 5.1). Another study observed that the ratio of WNME to infection was 1:50 in persons aged $\geq$65 years, and 1:300 in persons aged $<$65 (Mostashari *et al.*, 2001). Advanced age was also associated with disease severity among 19 hospitalized patients in 2000 (Weiss *et al.*, 2001).

The large number of cases in 2002 provided a better understanding of the effect of age on the risk of development of both WNME and WNF. Whereas 36% of WNME cases were less then 50 years old, 55% of WNF cases met this criterion. The mean age of WNF cases was 48 years compared with 59 years for WNME. Youth seems to protect from development of severe disease after infection with WNV (CDC, 2002k).

Mostashari *et al.* (2001) attempted to identify risk factors of human infection (not disease) through questions that were administered to healthy subjects living in Queens in October 1999. From these questions, and seroprevalence status, the risk factors that emerged were: time outdoors when mosquitoes were biting, and presence of dead birds in the neighborhood. For those who spent time outdoors, use of mosquito repellent had a protective effect. Another study implicated vegetation cover as linked with WNV risk in humans during the 1999 outbreak (Brownstein *et al.*, 2002).

Because WNV is known to cause viremia in humans, blood transfusion was considered a potential risk factor for WNV infection after the 1999 epidemic in New York City. The theoretical risk of transmission from donors was estimated at 1.8:10,000 (Biggerstaff and Petersen, 2002). In 2002 the first cases of transfusion transmission were documented. These and other cases that occurred due to transmission by means other than mosquito bite are discussed later (see Section VII,C). Other risks that emerged in 2002 besides infection by mosquito bite included organ transplantation, pregnancy (risk to developing fetus), breastfeeding (risk to infant), and occupation (laboratory workers that contact WNV directly). No risk of WNV infection has been described for the following potentially risky behaviors: caring for human cases, sexual contact, bird feeding, handling live birds or other vertebrates, eating bird-derived foods, and handling of infected animal carcasses (outside the laboratory). Presumably risk of WNV infection through these behaviors is exceedingly low, or possibly overlooked.

Risk factors for WNV infection in North American horses were evaluated by a case control study conducted at 150 horse premises in 2000 (USDA, 2001). Proximity to communal bird roosts or waterfowl congregations, and dead birds noted on premises were more frequent in case premises relative to controls, but these associations were not statistically significant. Age and gender were not risk factors for either infection or disease in horses.

Risk of local transmission was evaluated early in 2000 when the significance of finding a WNV-positive dead crow was not yet understood (Nasci *et al.*, 2002). In three locations around New York City in May–July, 2000, where single dead WNV-positive crows were detected, other indicators of local transmission were also present, including WNV-infected *Culex* mosquitoes (in all three locations), and seropositive immature house sparrows (in one of the three locations).

The significance of the finding of WNV-positive dead birds as a risk factor for human disease has been the subject of much debate. The initial observation that many counties with WNV-positive dead birds did not report human cases suggested that this finding was a poor predictor for human infections (but clearly an indicator that the primary bird-mosquito WNV transmission cycle was active) (Eidson *et al.*, 2001a, 2001b). However, a recent analysis of data from 2001 found that a single WNV-infected dead crow early in the transmission season (before August 5) indeed indicated elevated risk (relative risk 6.4) of human cases (Guptill *et al.*, 2003). Preliminary analysis of the 2002 data determined a relative risk of 2.4. However, this risk varied regionally. Density of reported dead crows was evaluated as a potential risk factor for human cases in 2000 in the northeast United States (Eidson *et al.*, 2001c; Hadler *et al.*, 2001; Julian *et al.*, 2002). Weekly dead crow densities above 0.6 per square mile predicted the appearance of human cases in the four New York state counties where cases occurred. These observations need further corroboration. In 2001, complex GIS software programs designed to detect clusters of dead birds in space and time successfully predicted locations of future human WNME cases in New York City (Mostashari *et al.*, 2003; Theophilides *et al.*, 2003).

VII. Ecology

A. Invertebrate Hosts (Vectors)

Important mosquito vectors for WNV in Europe, Africa, the Middle East and Asia are various ornithophilic members of the *Culex* genus, including *Cx. tritaeniorhynchus* in south Asia, *Cx. annulirostris*

in Australia, *Cx. perexiguus* (formerly *Cx. univittatus*) in North Africa and the Middle East, *Cx. univittatus* in sub-Saharan Africa, and Old World forms of *Cx. pipiens* and *Cx. quinquefasciatus* throughout the regions where their ranges overlap with the distribution of WNV activity (Komar, 2000). The 1996 Bucharest outbreak was driven by *Cx. pipiens* (Savage *et al.*, 1999). Numerous other mosquito species have been found infected, but in general the infection rates in these species have been low. Similarly, WNV isolates have been made from several species of ticks belonging to the families Ixodidae and Argasidae, but none of these other mosquitoes or ticks are thought to be vectors of important consequences to public health (Hayes, 1989). In North America, data on vectors have come from both field and laboratory studies.

1. Field Studies

In New York City, the outbreak investigation of September 1999 yielded 15 isolates from *Culex* species mosquitoes, including *Cx. pipiens*, *Cx. salinarius*, and *Cx. restuans* (Nasci *et al.*, 2001a). Minimum infection rates (MIRs) in each of these species could not be derived because many of the pools of *Culex* mosquitoes that were tested carried more than one species of *Culex*. The overall MIR derived for all *Culex* was 3.1 per 1000 mosquitoes tested. In contrast, no isolates were made from 3274 *Ochlerotatus triseriatus* or from 7956 *Aedes vexans*, two abundant mammalophilic species. Similarly, higher infection rates were observed in *Culex* species relative to other species in Connecticut in 2000 (Andreadis *et al.*, 2001), New York State in 2000 (White *et al.*, 2001), and New York City in 2000 (Kulasekera *et al.*, 2001), although in 2000 locally high MIRs in *O. triseriatus* (5.0) and *O. japonicus* (0.7) occurred in Staten Island (Kulasekera *et al.*, 2001) and Orange County, NY (White *et al.*, 2001), respectively. *O. triseriatus* feeds primarily on small mammals, such as squirrels, suggesting that small mammals may be involved in a WNV transmission cycle. Although the Asian species, *O. japonicus*, is known to feed on mammals (including people), the identity of its preferred host in North America is unknown. This exotic species was recognized in the New York City region 1 year before the emergence of WNV, in 1998, and like WNV, has also spread rapidly from New York City (Fonseca *et al.*, 2001; Peyton *et al.*, 1999). Also in 2000, numerous WNV-infected *Cx. restuans* and *Cx. salinarius* pools were reported (Marfin *et al.*, 2001). As WNV spread southward and westward in the United States, additional *Culex* species mosquitoes became infected, including *Cx. nigripalpus* and *Cx. quinquefasciatus* in southeastern U.S. states (CDC, 2002a) and *Cx. tarsalis* in west-central states (CDC, 2002k). These three species are also important vectors for SLEV

(Tsai and Mitchell, 1988). Preliminary surveillance data for the United States in 2002 reported 4943 WNV-positive mosquito pools (representing 1.3 million mosquitoes tested), 55% of which were *Culex* mosquitoes (CDC, 2002k). Through 2002, 36 WNV-infected mosquito species had been reported in the United States. The role of most of these species in WNV transmission cycles has not yet been confirmed.

Culex spp. are also important in their potential role for overwintering WNV in temperate climates, where they hibernate as adult mosquitoes. Field evidence of this phenomenon was observed in the cold months of early 2000 when three WNV-infected hibernating adult *Cx. pipiens* mosquitoes were collected in Queens, New York City, near the epicenter of the 1999 outbreak (Nasci *et al.*, 2001b). In the fall, *Cx. pipiens* mosquitoes destined for hibernation undergo a developmental arrest (diapause) determined by the effect on the pupal stages of shortening day-length. The mosquitoes entering diapause feed only on plant sugars and do not blood-feed, so presumably the overwintering mosquitoes acquired their infection by vertical transmission, which is discussed later (see Section VII,C).

Bloodfeeding patterns of mosquitoes are important for understanding the vector potential of different species. Apperson *et al*. (2002) analyzed 256 engorged mosquitoes (including 185 *Culex* species mosquitoes) collected from parks around northeast Queens, NYC, during the summer following the 1999 outbreak. *Cx. pipiens* and *Cx. restuans* were predominantly ornithophilic (bird:mammal ratios 23:1 and 6:1, respectively) while *Cx. salinarius* was predominantly mammalophilic (ratio 1:4). These observations favored a bridge (bird-to-mammal) vector role for *Cx. salinarius*, but indicate that the ornithophilic *Culex* species may be responsible for many mammalian infections as well. The avian blood meals identified to species from *Cx. pipiens* ($n = 38$) were mostly from American robins (16%), northern cardinals (13%), and northern mockingbirds (13%). The absence of identified blood meals from corvids, pigeons, house sparrows, and waterfowl was surprising, as the avian mortality surveillance (Eidson *et al.*, 2001a) and the seroprevalence studies (Komar *et al.*, 2001a) from the same locations indicated high WNV infection rates in these species. Corvids may have been locally extirpated by WNV during the period of the study. More blood meal analyses from a wider range of habitats are needed.

2. Laboratory Studies

Initial vector competence studies on field-collected mosquitoes from New York revealed that *Cx. pipiens* and *A. vexans* were moderately efficient vectors, although this efficiency was dependent on the dose

of virus imbibed (Turell *et al.*, 2000). When *Cx. pipiens* mosquitoes fed on viremic blood containing $10^{5.2}$ pfu/mL, only 2% of these mosquitoes were able to transmit after a period of extrinsic incubation. However, if the infecting dose concentration were increased to $10^{7.0}$, 20% of mosquitoes transmitted. Expanded studies by Turell and colleagues evaluated 13 additional species for vector competence (Sardelis and Turell, 2001; Sardelis *et al.*, 2001, 2002; Turell *et al.*, 2001a). *Culex* species were moderately competent (when exposed to a viremia of 10^7 pfu/mL), whereas certain container-breeding species (e.g., *A. albopictus*, *O. japonicus*) were most competent, and floodwater-breeding species (e.g., *A. vexans*, *A. taeniorhynchus*) were least competent (Table VI). However, competence is only one factor that contributes to the importance of mosquitoes as vectors. When other factors are considered, such as mosquito

TABLE VI

Estimated Vector Competence of Selected North American Mosquito Species for West Nile Virus, Based on Ingestion of a Bloodmeal Containing Approximately 10^7 pfu/mL and 12–15 Days Extrinsic Incubation

Species	*n*	Infection Rate[a]	Transmission Rate[b]
Culex erythrothorax	25	100	64
Cx. nigripalpus	127	84	10
Cx. pipiens	209	84	25
Cx. quinquefasciatus	236	63	30
Cx. salinarius	20	95	35
Cx. stigmatosoma	48	77	19
Cx. tarsalis	91	81	62
Ochlerotatus dorsalis	29	41	34
O. japonicus	119	76	71
O. melanimon	60	48	20
O. sierrensis	50	14	6
O. taeniorhynchus	75	12	3
Aedes albopictus	241	81	66
A. sollicitans	50	70	12
A. vexans	35	37	17
Culiseta inornata	28	75	21

[a] Percentage of mosquitoes exposed per os that become infected.

[b] Estimated percentage of mosquitoes exposed per os that are competent to transmit by bite.

From Goddard *et al.*, 2002; Sardelis and Turell, 2001; Sardelis *et al.*, 2001, 2002; Turell *et al.*, 2000, 2001a.

densities, host-feeding preferences, feeding behavior, and seasonal activity, the ornithophilic *Culex* species are implicated as most important enzootic vectors, whereas *A. albopictus*, *O. japonicus*, and *O. triseriatus* are suspected bridge vectors (Turell *et al*., 2001b, 2002). *Cx. salinarius* is likely an important bridge vector as well, due to its catholic feeding behavior (Apperson *et al*., 2002; Kulasekera *et al*., 2001).

In preparation for WNV's arrival in California, researchers there evaluated the vector competence of several California mosquito populations, including 10 species (Goddard *et al*., 2002). In accordance with previous studies, they found that all mosquitoes tested were competent to some degree, and that competence varied widely. Interestingly a significant difference was found in susceptibility to infection among *Cx. quinquefasciatus* derived from extreme southern California compared with the same species derived from Bakersfield in the Central Valley (see also Sardelis *et al*. [2002] for a description of competence variation among strains of *A. albopictus*). Transmission rates increased among all species when the extrinsic incubation period (the interval between feeding on a blood meal and the test for transmission) was increased from 7 to 14 days. Consistent with other studies, infection rates were reduced when the initial dose was decreased to about 10^5 pfu/mL. However, *Cx. tarsalis*, *Cx. pipiens*, *Cx. stigmatosoma*, and *Cx. erythrothorax* transmission rates were 82%, 60%, 34%, and 30%, respectively, after feeding on this dose, suggesting that thresholds for infection of these populations are significantly less than 10^5 pfu/mL. These transmission rates were determined by detecting virus particles in mosquito saliva after 14 days of incubation.

The differences found in the transmission rates for *Cx. pipiens* mentioned previously could be related to environmental temperature, since this variable is well known to affect flavivirus replication in mosquitoes during the extrinsic incubation period (Hess *et al*., 1963; Whitman, 1937). The New York mosquitoes were incubated at 26 °C, compared with 28 °C for the California mosquitoes. Increased temperature does increase the vector competence of *Cx. pipiens* (Dohm *et al*., 2002) and *Cx. univittatus* (Cornel *et al*., 1993).

The role of non-culicine arthropods, such as ticks, lice, mites, fleas, tabanids, etc., in the transmission cycle of WNV in North America is currently unknown. Hayes (1989) considered ticks as potential vectors. Several isolates were reported from *Argas*, *Hyalomma*, and *Ornithodoros* species ticks, but ecologic data did not suggest an important role for ticks. However, experimental infection studies demonstrated vector competence of four species of *Ornithodoros* soft ticks. In experimental studies, four North America tick species were found incompetent,

including *Amblyomma americanum*, *Dermacentor variabilis*, *D. andersoni*, and *Ixodes scapularis*. Transtadial transmission occurred from larvae to nymphs in the latter three species, but they failed to transmit to mice or hamsters (Anderson *et al.*, 2003). The only other evidence for non-culicine vectors in North America was the isolation of WNV from a pool of blood-engorged ectoparasitic louseflies (Diptera: *Hippoboscidae*) collected from a WNV-positive great horned owl in Pennsylvania (M. Hutchinson, personal communication). The vector competence of this fly is unknown.

B. Vertebrate Hosts (Reservoirs)

Birds are the primary vertebrate hosts for WNV in the Old World (Hayes, 1989). This was determined by extensive serosurveys of birds and mammals in several locations, including Egypt (Taylor *et al.*, 1956), Israel (Akov and Goldwasser, 1966), South Africa (McIntosh *et al.*, 1968), Pakistan (Hayes *et al.*, 1982), Romania (Savage *et al.*, 1999), and others. In these endemic/enzootic regions, birds were frequently infected (as determined by presence of antibodies) and experimental infection studies confirmed that some birds developed high levels of viremia. Essentially all vertebrate hosts that were exposed, whether by inoculation or by infectious mosquito bite, developed viremia and/or raised antibodies. However, birds stand out from other vertebrates as being important WNV amplification hosts due to the development of viremias of sufficient duration and magnitude to infect vector mosquitoes (Fig. 3). Other vertebrates are rarely involved in transmission cycles.

New information on vertebrate hosts of WNV in North America has mostly derived from experimental infection studies with the New York 1999 strain of WNV and field studies in New York State.

1. Field Studies

Komar *et al.* (2001a, 2001b) evaluated WNV exposure of domestic and peridomestic birds and mammals after the 1999 epidemic in New York City. A total of 430 live birds of 18 species were sampled and 33% had neutralizing antibody to WNV. Birds were sampled in four boroughs of New York City and two adjacent counties. The seroprevalence rates in these locations radiated outward from the epicenter in the borough of Queens, where the most human cases were recorded. Here the seroprevalence in birds reached 50% (compared with 2.6% in people). The relative importance of different species was analyzed for Queens by combining seroprevalence and relative abundance data (Table VII).

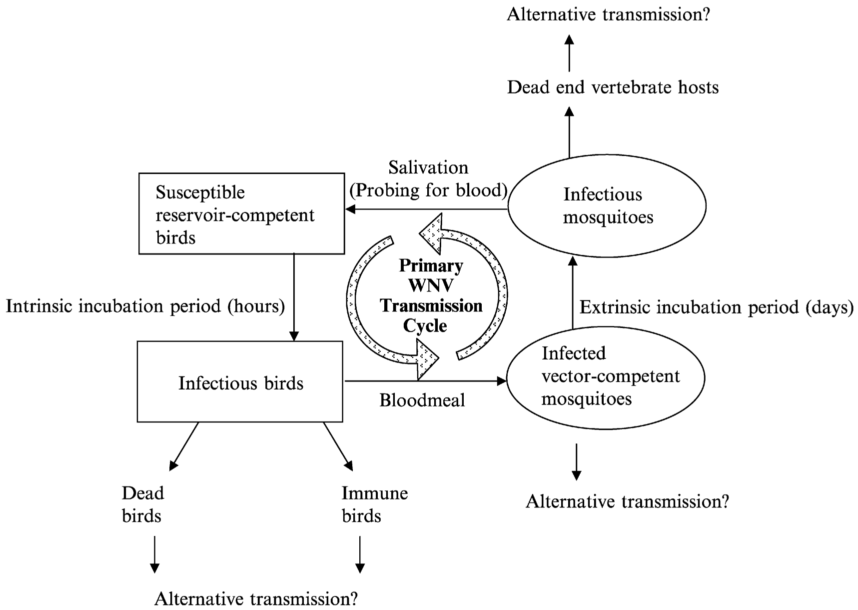

FIG 3. The primary West Nile virus transmission cycle involves certain reservoir-competent birds that transmit virus to feeding mosquitoes during a brief period of elevated viremia that follows infection. Viremia is rapidly neutralized by development of antibodies. A portion of vector-competent mosquitoes that survive the extrinsic incubation period may transmit to susceptible birds to keep the cycle going. Alternative modes of transmission may exist at multiple points along the cycle.

TABLE VII

RELATIVE ABUNDANCE, SEROPREVALENCE ("INFECTION RATE"), AND RELATIVE NUMBER OF WEST NILE VIRUS INFECTIONS AMONG BIRDS SAMPLED IN QUEENS, NEW YORK CITY, SEPTEMBER 1999

Species	Relative Abundance	Infection Rate	Rel. No. Infections[a]
House sparrow	6000	0.60	4186
Pigeon	1000	0.27	314
Canada goose	60	0.29	20
Mallard	60	0.06	4
Chicken	3	0.63	2
Domestic goose	1	0.86	1

[a] Relative number of infections = relative abundance × infection rate.
From Komar *et al.*, 2001a.

Infections in house sparrows occurred far more frequently than any other type of bird tested.

The seroprevalence of house sparrows in northeastern Queens was 60% in September 1999. In July 2000, seroprevalence in adult ("after hatch-year") house sparrows at the same location was still 60%, and approximately 1% of hatch-year birds were seropositive, suggesting that transmission was continuing in house sparrows in 2000 in spite of the high levels of background immunity in the adult population (Nasci *et al.*, 2002).

Mammals sampled in 1999 included horses, dogs and cats. None of 12 pet cats circulated antibodies. However, some dogs and horses were seropositive, with most of the infections detected from Queens, where one of 18 (5.6%) horses and six of 55 dogs (10.9%) were positive. The investigators concluded that because the infection rates in peridomestic mammals and birds were greater than that in humans in the epicenter of the outbreak, these animals may serve as useful sentinels.

A similar evaluation of birds (but not mammals) was conducted in the New York City borough of Staten Island after the epidemic there in 2000 (Komar *et al.*, 2001c). Transmission was focal throughout the island, with seroprevalence rates in house sparrows ranging from 0% to 25% among the nine study sites. Overall seroprevalence in house sparrows was low (9%), but these were still considered important hosts because of their abundance. Captive pigeons had a very high seroprevalence (54%), leading the researchers to speculate that pigeons in particular would make good sentinels. Free-ranging species with high seroprevalence included northern cardinal (69%), house finch (40%), and gray catbird (35%), all of which were residents on the island during the period of the study.

The seroprevalence in 257 resident birds in Staten Island was compared with that in 96 transient migrants. No seropositive migrants were detected, as expected, because migrants had arrived in Staten Island after the period of epizootic activity, and were unlikely to have come from other areas with intense WNV transmission. The exposure rate in resident species was 23%.

A role for migratory birds in the transmission cycle of WNV in North America has not been established. However, a recent Israeli study may shed some light on birds as dispersal vehicles for WNV. A mortality event in white storks was investigated near the city of Eilat on the Red Sea (Malkinson *et al.*, 2002). A flock of 1200 migrating storks arrived outside Eilat in August 1998. These storks do not usually pass through Eilat on their southward migration, but rather migrate across the Rift Valley either further north or further south. Their arrival in

Eilat was attributed to unusually strong westerly winds, which grounded the birds. Two days after arrival, 13 of these birds were observed ill and dead, and four WNV isolates were made from brain samples from these birds. Four days later, three of 11 healthy storks sampled in Eilat circulated antibodies indicating previous exposure of the flock to WNV. Because most of these birds were less than 1 year old, it was determined that exposure to WNV occurred earlier in the 1998 season, either in Europe or along the migration route. Sequence data from the stork isolates matched isolates made from other dead birds collected further north in Israel in 1998. Although the authors suggest that these storks present evidence that migrating birds, such as storks, disseminate WNV, the importance of this event is uncertain. For migrating storks to disseminate the virus, the virus would have to pass to other hosts along their migration route. However, no data are presented to indicate that the storks were sufficiently viremic to infect vector mosquitoes. Furthermore, although unlikely, it is possible that many of the dead storks, if not all of them, became infected in or near Eilat, where WNV may be endemic (Bin *et al.*, 2001). Because experimental pathogenesis studies in storks are lacking, the incubation period for WNV in storks is unknown. An unpublished report of a WNV isolate from 25 white storks sampled in 1998 further south in the Sinai Peninsula of Egypt, if corroborated, would strengthen the hypothesis that these storks carried the infection south on their migration (Malkinson and Banet, 2002).

Migrating birds have often been speculated as dispersal hosts for WNV (Malkinson and Banet, 2002; Malkinson *et al.*, 2002; Rappole *et al.*, 2000; Tsai *et al.*, 1998). While epizootiologic data collected in the field have not disproven this hypothesis, they have not definitively proven it either (Murgue *et al.*, 2002).

2. *Laboratory Studies*

Serologic studies in the field indicate which species are exposed to WNV infection (Komar, 2001). However, quantitative experimental data on viremia are needed to better understand which species are important reservoir hosts* from which blood-feeding mosquitoes may become infected. Such data from Egypt and South Africa confirmed the role of passerine birds as important reservoirs in those regions

* The term "reservoir host" as used herein refers to vertebrate hosts that are infectious to vector mosquitoes. The concept of reservoir host can be confusing because mosquitoes may harbor arbovirus infections for a longer time than the vertebrate hosts that infected them.

(McIntosh *et al.*, 1969; Work *et al.*, 1955). Similarly, numerous experimental infection studies of mammals using Old World WNV strains have indicated that most species, albeit susceptible, are incompetent as reservoir hosts (reviewed in Hayes, 1989; Komar, 2000).

Experimental infection studies with the New York 1999 strain of WNV have now been published for 28 species of birds and three species of mammals. Senne *et al.* (2000) and Langevin *et al.* (2001) evaluated chickens as hosts. Both studies found ephemeral, low level viremias, with maximum viremia not exceeding 10^5 pfu/mL, indicating that chickens are relatively ineffective sources of infection for most mosquito vectors. Both studies found that chickens shed low amounts of WNV per cloaca, and Langevin *et al.* found that low-level shedding also occurred per os. Langevin *et al.* concluded that chickens were generally safe to use in arbovirus surveillance programs as sentinels, although biosafety precautions were recommended for handlers.

Swayne *et al.* (2000, 2001) evaluated four domestic geese and eight turkeys as hosts for WNV. Young goslings and poults were used, and in both cases viremias lasted longer and reached higher maxima compared with chickens. However, maximum viremias ($10^{7.5}$ $TCID_{50}$/mL in goslings; $10^{5.5}$ $TCID_{50}$/mL in poults) were only weakly infectious for *Cx. pipiens* mosquitoes. Low-level cloacal shedding (but not oral) was observed in poults, and low-level oral shedding (but not cloacal) was observed in goslings. Fatal pathology occurred in three of four goslings. The authors concluded that goslings but not poults were competent reservoir hosts.

Komar *et al.* (2003a) evaluated 25 species of birds, including domestic and free-ranging species, as WNV hosts. Reservoir competence index values (Table VIII) were calculated for each of the species, based on a formula that was developed for work with eastern equine encephalitis virus (Komar *et al.*, 1999). The values were derived from viremia profiles. The competence index indicates the relative number of infectious *Cx. pipiens* mosquitoes that would derive from feeding on an average infected vertebrate host, assuming each received the same number of bites. Passerine species scored highest, although fish crow and European starling had relatively low competence values. Ring-billed gull and killdeer, both charadriiforms, had high competence scores, mainly due to long-lasting viremias, making these long-distance migrants candidates for important WNV dispersal hosts. Great horned owl and American kestrel also scored high.

Several orders of birds were incompetent in this study including Piciformes (a woodpecker), Psittaciformes (parakeets), and Galliformes (quail, pheasant). Anseriformes (ducks and geese), Gruiformes

TABLE VIII
COMPUTATION OF RESERVOIR COMPETENCE INDEX VALUES FOR SELECTED BIRD SPECIES FOR TRANSMISSION OF WEST NILE VIRUS TO MOSQUITOES[a]

Species (n)	s	i	d	c_i
Blue jay (4)	1.0	0.68	3.75	2.55
Common grackle (6)	1.0	0.68	3.0	2.04
American crow (8)	1.0	0.50	3.25	1.62
House sparrow (6)	1.0	0.53	3.0	1.59
Ring-billed gull (2)	1.0	0.28	4.5	1.26
American kestrel (2)	1.0	0.31	3.0	0.93
Great horned owl (1)	1.0	0.22	4.0	0.88
Killdeer (2)	1.0	0.29	3.0	0.87
Fish crow (9)	1.0	0.26	2.8	0.73
European starling (6)	1.0	0.12	1.8	0.22
Canada goose (3)	1.0	0.10	0.3	0.03
Rock dove [pigeon] (6)	1.0	0.00	0.0	0.00
Budgerigar [parakeet] (3)	0.7	0.00	0.0	0.00

[a] Each value (c_i) was derived by taking the product of s, the proportion of hosts that were susceptible to infection by mosquito bite, i, the mean infectiousness to *Culex pipiens* mosquitoes (a value derived from viremia titers), and d, the mean duration (in days) of infectious-level viremias (Komar *et al.*, 2003a).

(a coot), and Columbiformes (doves) were weakly competent, although pigeons were incompetent. These groups of birds are unlikely to be important reservoir hosts.

Viral shedding was evaluated in most of these bird species, and both oral and cloacal shedding was confirmed in the majority. In general, shedding was low and considered inconsequential. However, several species, particularly passerines and owls, shed large quantities of virus (up to $10^{6.4}$ pfu/swab), suggesting that shedding could be a source of infection for contacts, even human handlers. Furthermore, swabs of oral and cloacal cavities of corvids that died were consistently high titered, indicating oral and cloacal swabs as a source of diagnostic specimens easily obtained from carcasses (Komar *et al.*, 2002).

Interestingly, infectious WNV was detected in tissues of some surviving birds up to 13 days after viremia was no longer detectable (Table IX), suggesting possible long-term persistence in birds. Chronic WNV infections, as well as other flavivirus infections, have been documented previously (reviewed in Kuno, 2001a). Birds that no longer have viremias but contain viable virus in tissue may be the source of

TABLE IX

WEST NILE VIRUS QUANTITIES DETECTED IN TISSUES FROM SELECTED BIRDS AT 14 DAYS POST-INFECTION BY MOSQUITO BITE[a]

Bird	Tissue	Viral Load	Days Post-viremia
Killdeer 1	Skin	110 pfu/0.5 cm^3	9
Killdeer 2	Spleen	550	10
Killdeer 2	Skin	20,000	10
Mourning dove	Kidney	100	11
Budgerigar	Heart	130	13
Blue jay	Eye	360	9
Common grackle	Skin	380	11
Common grackle	Eye	150	11
House sparrow 1	Skin	370	8
House sparrow 2	Spleen	120	10
House sparrow 2	Lung	590	10
House sparrow 3	Brain	300	8

[a] Only tissues with viral loads of $\geq$100 pfu/0.5 cm^3 are presented. Negative results are not shown.

From Komar *et al*., 2003a.

oral infection of predators but would not be expected to transmit to mosquitoes through bloodmeals (see Section VII,C).

A study of horses was conducted to better understand the pathogenesis of WNV in this susceptible host (Bunning *et al*., 2002). Horses were incompetent reservoir hosts, with a maximum viremia of $10^{3.0}$ pfu/mL, well below the threshold for infecting vector mosquitoes. To be sure, 652 *A. albopictus* mosquitoes were fed on horses circulating as much as $10^{2.7}$ pfu/mL and incubated for 7 days before testing for infection. None of the mosquitoes became infected.

Two studies of small rodents found that laboratory mice and hamsters may indeed develop infectious level viremias. Kramer and Bernard (2001) showed that detectable viremia endured 3–4 days in Balb/C mice (*Mus musculus*) after intraperitoneal inoculation, reaching a maximum of $10^{5.4}$ pfu/mL. Golden hamsters (*Mesocricetus auratus*) developed peak viremias between 10^5 and $10^{5.8}$ $TCID_{50}$/mL (Xiao *et al*., 2001). However, viremia profiles of naturally occurring North American rodent species have not been studied.

There are rare occasions in which non-avian hosts may function as reservoir hosts. For example, Malagasy lemurs (*Lemur fulvus*) infected with a lineage 2 Madagascar strain of WNV transmitted the infection

to *A. aegypti* mosquitoes and were suspected as reservoir hosts in Madagascar (Rodhain *et al.*, 1985). Lake frogs (*Rana ridibunda*) inoculated with a Russian strain of WNV developed viremia as high as $10^{5.7}$ $SMLD_{50}$/mL and transmitted the virus to *Cx. pipiens* (Kostiukov *et al.*, 1986). Isolation of WNV from free-ranging frogs suggested involvement in a transmission cycle in Russia (Kostiukov *et al.*, 1985). Late in 2002, WNV was isolated from captive American Alligators (*Alligator mississippiensis*) in Georgia (Miller *et al.*, 2003). Post-mortem serum samples from captive alligators in Florida revealed high-titered viremias suggesting that this species may be a competent vertebrate reservoir for WNV (CDC, unpublished data). The importance of non-avian vertebrates for WNV in North America requires more study.

C. Alternative Modes of Transmission

Potential alternative modes of transmission for WNV include vertical transmission and direct contact transmission between vertebrate hosts in the absence of arthropod vectors. The latter category would include sexual, fecal-oral, bloodborne, oral, and aerosol transmission. Kuno (2001b) reviewed potential alternatives to mosquito-borne transmission for WNV and other arboviruses.

Vertical transmission of WNV has been reported for both vertebrates and invertebrates. In 2002, one case of WNV infection in an infant in the United States was attributed to transplacental transmission that occurred subsequent to a mosquito-borne infection in August in a 20-year-old pregnant woman (CDC, 2002m). The infant was born with severe brain damage approximately 11 weeks later (at full term) in November. Anti-WNV IgM was present both in blood and CSF, and placental tissue was positive for WNV RNA. Vertical transmission in non-human vertebrates has not been reported. However, the report of a low-level persistent infection in the ovary of a common grackle 11 days after termination of detectable viremia raises the question whether transovarial transmission in birds may be possible (Komar *et al.*, 2003a). Transplacental transmission in mice (Mathur *et al.*, 1982), pigs (Burns *et al.*, 1950), and humans (Chaturvedi *et al.*, 1980) has been reported for JEV.

Transovarial transmission of WNV is known to occur in mosquitoes. Transmission to adult F1 progeny occurred at a low rate (1:325) in *Cx. tritaeniorhynchus* mosquitoes that were inoculated intrathoracically, and at moderate rates in *A. albopictus* (1:124) and *A. aegypti* (1:62) (Baqar *et al.*, 1993). Low rates of transovarial transmission were observed in intrathoracically inoculated *Cx. pipiens,* with minimum filial

infection rate of 0.6 per 1000 (Turell *et al.*, 2001a) and 2.1 per 1000 (Dohm *et al.*, 2002). In the latter study, none of over 13,000 adult progeny of infected *A. albopictus* mosquitoes were infected. These investigators speculated that vertically infected mosquitoes that hibernate during winter reinitiate primary transmission cycles in the spring. Natural evidence for vertical transmission was obtained from the isolation of WNV from a pool of male *Cx. univittatus* in Kenya (Miller *et al.*, 2000) and from hibernating female *Culex* spp. mosquitoes in New York City (Nasci *et al.*, 2001b).

The apparent die-offs observed in domestic geese in Israel and free-ranging corvids in the United States, both of which began in the late 1990s, suggested that alternative modes of transmission other than mosquitoes might exist among birds for these new aviopathic strains of WNV. Six separate experimental infection studies in the United States evaluated direct transmission among cage contacts. Langevin *et al.* (2001) and Senne *et al.* (2000) placed uninfected chickens together in cages with infected chickens. One cage mate (of 16 exposed) in Langevin's study became infected in the absence of mosquitoes. The mode of this "cage mate transmission" was not determined, although low-level shedding of WNV per cloaca (as well as per os) was observed in some of the chickens, suggesting the possibility of fecal-oral transmission. Orally challenged chickens did not become infected (Langevin *et al.*, 2001). One cage mate domestic gosling also became infected (of two exposed), and low-level shedding per os (but not per cloaca) in three of four infected goslings was also detected (Swayne *et al.*, 2001). Direct transmission was observed among needle-inoculated American crows held together in an indoor aviary (McLean *et al.*, 2002). The same observation was made among mosquito-inoculated American crows held in cages with contact controls (Komar *et al.*, 2003a). In this study, four cages each held two infected and one uninfected crow, except for one cage that held two uninfected crows. Viremias were monitored daily in these birds, and both the mosquito-inoculated birds and their contacts developed similar viremia profiles, with onsets in the contacts typically occurring about 1 day after the infected cagemates died from the infection (Fig. 4). In the cage with two contacts, the onset of the second contact occurred shortly after the death of the first contact control bird, suggesting that in this cage, transmission occurred from crow to crow to crow. Komar *et al.* (2003a) evaluated 17 other species of birds for direct contact transmission in the laboratory, and observed transmissions in three of these species, including blue jay, black-billed magpie, and ring-billed gull (Table X). The occurrence of direct contact transmission among birds in nature

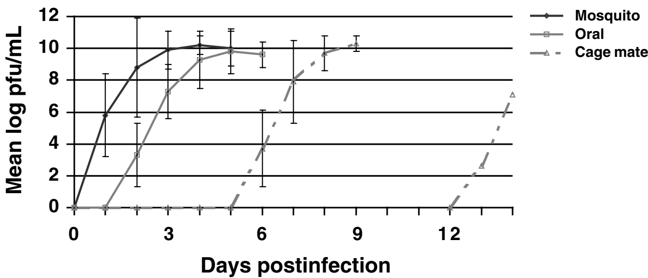

FIG 4. Viremia profiles in American crows infected by mosquito ($n = 8$) or by ingestion of infected bird carcasses ($n = 5$) or by direct contact with other infected crows ($n = 4$). Error bars show standard deviation of log-transformed viremia titers (Komar *et al.*, 2003a).

TABLE X

DIRECT TRANSMISSION OF WEST NILE VIRUS IN CAPTIVE BIRDS[a]

Species	No. Cages	No. Mosquito-Exposed Birds	No. Contact-Exposed Cage Mates	No. Transmissions
American crow	4	8	5	5
Blue jay	2	2	2	2
Black-billed magpie	3	3	3	2
Ring-billed gull	1	2	1	1

[a] Uninfected birds (contact-exposed group) were placed within cages containing birds (of the same species) that were infected by mosquito bite (mosquito-exposed group). Transmission to uninfected cage mates was determined by development of viremia or seroconversion (Komar *et al.*, 2003a). Negative results are not shown.

has not been documented. However, the case of a fatal WNV infection in a red-tailed hawk in early February 2000, in Westchester County, New York, was speculated to have occurred in the absence of mosquito-borne transmission (Garmendia *et al.*, 2000). One proposed method was ingestion of infected prey.

Oral transmission in birds using the NY99 strain of WNV was evaluated in 16 species of birds (Komar *et al.*, 2003a; Langevin *et al.*, 2001; McLean *et al.*, 2002). Of these, five species were susceptible to infection through ingestion of WNV-contaminated material, including water, dead birds and mice, and infected mosquitoes (Table XI). These findings suggest the possibility of naturally acquired infections through ingestion of invertebrate or vertebrate prey items, or even contaminated

TABLE XI
ORAL TRANSMISSION OF WEST NILE VIRUS ACHIEVED UNDER EXPERIMENTAL CONDITIONS IN FIVE SPECIES OF BIRDS

Species	*n*	Dose[a]	No. Viremic
Common grackle	4	1000 pfu	4
House finch	1	mosquito	1
House sparrow	6	10^7 pfu	6
American crow	6	sparrow	5
American crow	3	10^7 pfu	3
Great horned owl	1	mice	1

[a] Oral doses of were given in liquid suspensions, in dead infected mosquitoes, or in dead infected carcasses (sparrows or mice). Negative results are not shown.
From Komar *et al.*, 2003a.

water (e.g., by fecal material). Although natural transmission per os has not been reported in birds, such transmission would be very difficult to distinguish from other modes of infection. However, a report of WNV infection in a suckling infant human being strongly suggests that natural oral transmission occurred in humans through the ingestion of breast milk containing virus (CDC, 2002f). The infection in the mother occurred after childbirth by transfusion of contaminated blood products, and subsequently breast milk tested positive for WNV RNA. The infant had minimal outdoor exposure, indicating the breast milk as the most likely source of infection. A second infant with similar exposure remained healthy (CDC, 2002g). Flaviviruses appear to have tropism for exocrine glands, such as salivary gland, mammary gland, mucus secreting cells, and pancreas (Harrison *et al.*, 1980; Monath *et al.*, 1983).

Blood-borne transmission has also been reported. A series of seven reports published by the Centers for Disease Control and Prevention and collaborators documented a complex web of WNV infections apparently contracted through the blood supply (CDC, 2002b, 2002c, 2002e, 2002g-j; Pealer *et al.*, 2003). At least six infections were confirmed in the United States in 2002. Transfused blood products that resulted in transmissions included fresh-frozen plasma and packed red blood cells. This was the first evidence of bloodborne transmission for WNV. The initial discovery of transfusion transmission came as a result of investigating four cases of WNV infection in humans, all of whom received organ transplants from the same viremic donor. The transplanted organs included liver, heart, and kidneys (Iwamoto *et al.*, 2002).

These were the first cases of WNV in humans resulting from organ transplantation. The donor had been infected by blood transfusion.

Additional human WNV infections occurred as a result of percutaneous exposure in two US laboratory workers (CDC, 2002l), serving as a reminder that exposure to high concentrations of WNV particles requires enhanced biosafety practices for laboratorians. Both of these incidents followed laceration of skin with contaminated sharp instruments. Historically, WNV infection of laboratory workers was one of the criteria that led to its designation as a BSL-3 agent (Anonymous, 1980).

Although not well documented, aerosol transmission may be another mode of infection of concern to laboratory workers and other potential vertebrate hosts of WNV. The finding of shedding of WNV in high concentrations from passerine and some other types of birds raises the possibility that aerosol transmission may in fact occur in nature or among handlers of infected birds, such as zoo keepers or wildlife rehabilitators.

Taken together, these observations of alternative modes of transmission that do not include arthropod vectors suggest that vectors are not the only means of transmission in nature, and that some of these alternative transmission routes may in fact have contributed to the rapid spread of WNV in North America.

VIII. Future Directions

WNV has become endemic in North America, causing disease in vertebrates annually since its arrival in 1999. Between 1999 and 2002, it flared up in numerous local epidemic/epizootic hot spots. Presumably it will continue to cause local epidemics in its continued spread within North America. Tropical regions of the Americas are presumably the next frontier, and WNV might eventually spread to tropical, subtropical and temperate regions of Central and South America. The public and veterinary health impacts in regions where WNV already exists in a quiet equilibrium, or where other closely related flaviviruses have already generated genetic resistance in vertebrate populations, remain to be determined.

Many questions yet exist on the basic ecology of WNV in North America, such as the epidemiologic significance of alternative transmission cycles, and the precise mode of geographic dissemination (and especially the role of migrating birds as dispersal vehicles). The early years of WNV's establishment on this continent are the time to attack these questions, as once new equilibria are reached, the presence of the virus may become cryptic like its cousin SLEV. The lessons learned during the next several years of study of WNV will have

far-reaching impacts on our preparations for defending against WNV in the coming years and other future arboviral invaders.

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ADVANCES IN VIRUS RESEARCH, VOL 61

DENGUE EPIDEMIOLOGY: VIRUS EPIDEMIOLOGY, ECOLOGY, AND EMERGENCE

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References

I. Introduction

Dengue is an expanding public health problem in the tropics and subtropics. Reports suggest 2.5 billion people are at risk for dengue (Gibbons and Vaughn, 2002; Gubler, 1998) with up to 100 million dengue virus infections each year (Halstead, 1988), and more than 60,000 reported deaths to date (Halstead, 1998). Dengue occurs in Central and South America, South and Southeast Asia, Africa, the Caribbean, and Pacific regions. While few autochthonous cases have been reported in the United States since the 1940s, there have been recent outbreaks in Texas, Florida, and Hawaii (Anonymous, 2002a; Gill *et al.*, 2000; Rawlings *et al.*, 1998). Given the current pandemic status of dengue, it is clear that vector control is not generally applied with

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success. Such efforts are feasible if applied energetically, as shown by the success of a wide variety of mosquito control efforts targeted specifically to dengue vectors with (Halstead, 1984) or without (Bang and Pant, 1972; Hart, 2000; Kay, 1987; Pontes *et al.*, 2000; Soper *et al.*, 1943) the use of persistent organochlorine insecticides such as DDT. No specific anti-viral therapy nor licensed vaccine exists. Prevention of dengue through widespread vaccination remains an important objective of the World Health Organization and the governments of dengue-endemic regions (Brandt, 1990).

II. History

David Bylon is sometimes credited with the first clinical description of Dengue fever (DF) (Carey, 1971). In 1779, Bylon observed an epidemic of febrile disease in Batavia, a disease he termed 'knokkelkoorts,' knuckle or joint fever (Pepper, 1941). A similar epidemic was occurring in Cairo at or about the same time (1779) and was described by August Hirsch in the "Handbook of Geographical and Historical Pathology" (Hirsch, 1883). However, both of these epidemics may have been more compatible with chikungunya than DF (Carey, 1971). Dr. Benjamin Rush provided one of the first in-depth descriptions of a DF epidemic after observing numerous patients during the 1780 outbreak in Philadelphia, Pennsylvania (Rush, 1789). "Break-bone fever" was the popular name applied to this epidemic.

The term "dengue" is derived from the Swahili phrase "ka dinga pepo," where *ka* means "a kind of"; *dinga*, "sudden cramp-like seizure"; and *pepo*, "plague." This phrase is believed to have crossed from Africa to the Caribbean in 1827. In Cuba this phrase was identified with the Spanish word "dengue" and came to represent the stiff gait of people afflicted with disease (more likely chikungunya at this time) trying to minimize the joint and muscle pain associated with movement. Documents from Spanish archives indicate the use of "quebranta huesos" (break-bone) by a physician in Puerto Rico to describe a febrile illness in 1771, and the use of the term "dengue" by the Queen of Spain in 1801 to describe an acute febrile illness with bone and joint pains, hemorrhage, and jaundice (Rigau-Perez, 1998).

Early research into the etiology of DF implicated bacteriological (Ashburn and Craig, 1907), protozoan (Graham, 1903), and spirochetal (Ashburn and Craig, 1907) causes. Ashburn and Craig provided evidence for the viral etiology of the disease, making dengue virus the second human viral pathogen identified after the yellow fever virus

(Ashburn and Craig, 1907; Reed *et al.*, 1901; Sabin, 1952). Siler, Hall, and Hitchens researched the role of *Aedes aegypti* in the development and transmission of dengue virus, building on the work of Graham in Lebanon (Graham, 1903; Siler *et al.*, 1926). Research performed by Hotta and Kimura and Sabin and Schlesinger during World War II isolated virus types 1 and 2, identified the presence of homotypic immunity following infection, and described the clinical and diagnostic significance of neutralizing antibodies (Hotta, 1952; Sabin, 1952). In 1956, a dengue epidemic occurred in Manila resulting in the identification and naming of DEN-3 and DEN-4 viruses (Hammon *et al.*, 1960).

Dengue hemorrhagic fever (DHF), a severe form of dengue, has been widely recognized since dengue virus was associated with Philippine hemorrhagic fever and Thai hemorrhagic fever in the 1950s (Halstead *et al.*, 1963; Hammon *et al.*, 1960). In 1897, North Queensland reported a dengue outbreak with hemorrhagic manifestations including epistaxis, hematemesis, and gingival bleeding (Hare, 1898). DHF was reported sporadically from the Americas (Honduras, Jamaica, Puerto Rico, Curacao) in the late 1960s and 1970s (Anonymous, 1990). The Cuban DHF outbreak in 1981 marked the start of severe dengue disease in the Americas (Kouri *et al.*, 1989). More than two million persons have developed DHF over the past 25 years (Brandt, 1990).

III. Clinical Description

The incubation period for dengue fever is typically 3–7 days (range, 3–14 days) (Ashburn and Craig, 1907; Innis, 1995). Most infections, especially in children younger than 15 years of age, are asymptomatic or minimally symptomatic (Burke *et al.*, 1988). Older children and adults who become ill sustain either a mild febrile syndrome or a self-limited but incapacitating illness called dengue fever manifesting variably as fever (102–105 °F) lasting 2–7 days, myalgias, frontal headache, retro-orbital pain, nausea, vomiting, anorexia, altered taste and olfactory perception, and malaise. Recovery can be prolonged with an asthenia that can last for many weeks (Rigau-Perez *et al.*, 1998; Rush, 1789).

Physical examination may reveal paradoxical bradycardia, lymphadenopathy, conjunctival injection, inflamed pharynx, early or late rash, and facial flushing. The presence of petechial hemorrhage, a positive tourniquet test, and easy bruisability are suggestive of a dengue virus infection. The presence of hepatomegaly further supports the diagnosis (Nimmannitya *et al.*, 1969).

Laboratory findings include marked leukopenia and, commonly, thrombocytopenia (<100,000/mm^3) (Ashburn and Craig, 1907; Hayes *et al.*, 1988). Although rarely reported, hyponatremia was seen in 60% of 18 Israeli travelers who contracted DEN-1 from either Thailand or India (Schwartz *et al.*, 1996).

Disease may progress beyond the acute febrile stage (first 3–5 days) to a plasma leakage stage with or without significant hemorrhage that can result in shock or even death (Isarangkura *et al.*, 1987). This form of dengue virus infection, known as DHF and in cases of shock, dengue shock syndrome (DSS), have been most thoroughly studied in Southeast Asian children (Halstead, 1988). The time of defervescence represents a critical stage when more severe disease manifestations become evident. Warning signs for impending shock include intense sustained abdominal pain, persistent vomiting, restlessness or lethargy, a sudden change from fever to hypothermia with sweating and prostration, cold extremities, a rapid and weak pulse, hypotension, and/or narrowed pulse pressure (Nimmannitya, 1987). A decrease in platelets with a contemporaneous rise in hematocrit accompanies the progression to DHF (Isarangkura *et al.*, 1987). Serum protein and albumin are low in some patients with DHF. Ascites and right-sided pleural effusions (Figs. 1, 2) occur in nearly all cases complicated by shock (Kalayanarooj *et al.*, 1997). Abnormal hemostasis and disseminated intravascular coagulation (DIC) may occur and correlate with disease severity, although precise mechanisms have yet to be elucidated (Funahara *et al.*, 1983; Krishnamurti *et al.*, 2001; Srichaikul *et al.*, 1977; Wills *et al.*, 2002). Petechiae, easy bruising, bleeding at the site of venipuncture, and purpura/ecchymoses are common hemorrhagic manifestations. Gastrointestinal hemorrhage also occurs and can be fatal (George and Duraisamy, 1981; Sumarmo *et al.*, 1983).

The spectrum of DHF is classified into grades I through IV based on the severity of infection and the presence of hemorrhage and/or shock (Nimmannitya *et al.*, 1969). Grade I includes fever, nonspecific constitutional symptoms, evidence of plasma leakage without shock, and a positive tourniquet test as the only hemorrhagic manifestation. Grade II is the same as grade I with spontaneous hemorrhagic manifestations. Grade III represents circulatory failure manifested by a rapid, weak pulse, narrowing of pulse pressure (20 mm Hg or less), or hypotension. Grade IV is characterized by profound shock with undetectable pulse and blood pressure. DSS is considered grades III and IV (Anonymous, 1999).

Atypical manifestations of dengue virus infection include fulminant hepatitis, cardiomyopathy, acute renal failure, and encephalopathy

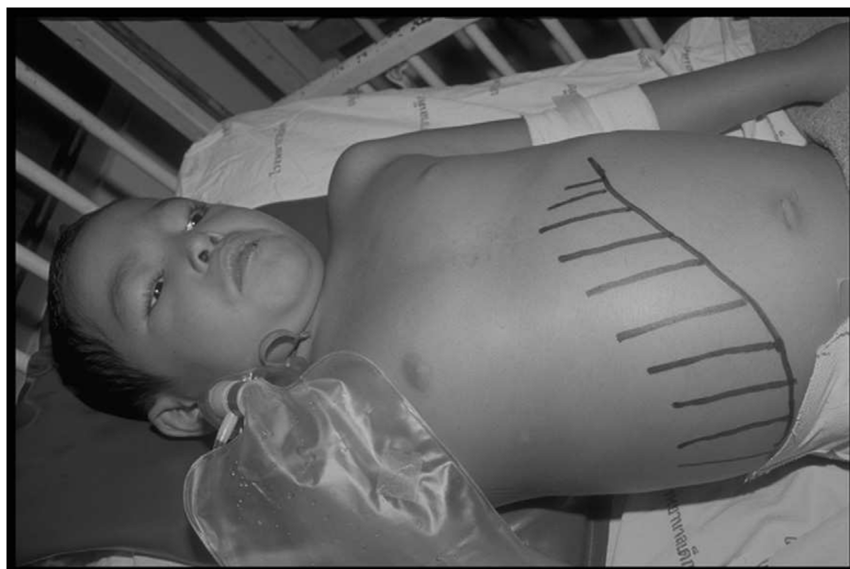

FIG 1. Dengue hemorrhagic fever patient with hepatomegaly (*markings on abdomen*), ascites as indicated by distended abdomen, and pleural effusion–induced need for supplemental oxygen. Used with permission of Dr. Siripen Kalayanarooj. (See Color Insert.)

(Nimmannitya *et al.*, 1987). Central nervous system manifestations may include altered consciousness, convulsions, and flaccid paralysis (Solomon *et al.*, 2000). Additional, albeit rare, clinical manifestations of dengue infections that have been reported include orchitis, ovaritis, keratitis, retinitis, acoustic disturbances, and cerebral edema (Hotta, 1969; Janssen *et al.*, 1998).

While congenital anomalies due to dengue virus infection in the mother have not been reported (Figueiredo *et al.*, 1994), rare cases of perinatal DF in the mother prepartum and neonate postpartum have been reported, suggesting that the virus can cross the placenta in utero (Boussemart *et al.*, 2001; Bunyavejchevin *et al.*, 1997; Chye *et al.*, 1997; Thaithumyanon *et al.*, 1994).

The treatment of DHF is a combination of prompt diagnosis, frequent assessment of intravascular and extravascular volume status, monitoring for hemorrhagic complications, and providing supportive care for the same.

Fluid resuscitation to counteract massive plasma leakage is the mainstay of treatment. Early and effective replacement of plasma losses results in a favorable outcome in most cases. Quickly reversing

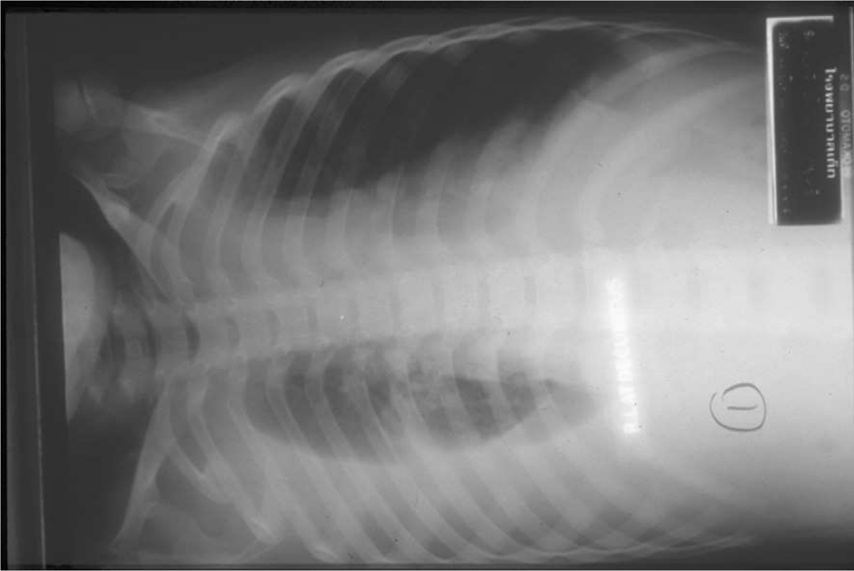

FIG 2. Pleural effusion in right lateral decubitus chest radiograph in child with dengue hemorrhagic fever. Used with permission of Dr. Siripen Kalayanarooj. (See Color Insert.)

shock and correcting electrolyte disturbances will help prevent the onset of disseminated intravascular coagulation (DIC). Colloid solutions (Dextran) appear to restore cardiac index and blood pressure and normalize hematocrit more rapidly than crystalloids (Ringer's lactate) (Dung *et al.*, 1999). The benefit of adjunctive steroids for the treatment of DHF has yet to be validated (Min *et al.*, 1975b; Pongpanich *et al.*, 1973; Sumarmo *et al.*, 1982; Sumarmo *et al.*, 1975; Tassniyom *et al.*, 1993).

Failure to clinically improve or the persistence of shock may indicate internal bleeding and fresh whole-blood transfusions may be required. Fresh-frozen plasma or platelets may also be considered in cases where DIC or other coagulopathies complicate the clinical course (Anonymous, 1997). The mortality rate in cases complicated by hemorrhage is three to four times greater than that in those without bleeding manifestations (Netrasiri *et al.*, 1966). The Queen Sirikit National Institute of Child Health (formerly the Bangkok Children's Hospital) has seen a steady decline in case-fatality rates, from approximately 10% in 1970, to 2% in 1984, to 0.2% in 1990 (Nimmannitya, 1997). Mortality is usually linked to delayed provision of supportive treatment and/or pre-morbid chronic illness (Anonymous, 1997).

Antipyretic drugs may be useful to reduce the risk of febrile convulsions in children. Salicylates should be avoided to reduce the risk of acidosis and bleeding complications. Paracetamol, dosed according to age, is preferred (Anonymous, 1997).

Dengue virus infections are rarely fatal in adults, although fatal infections do occur (Guzman *et al.*, 1999b). Autopsy studies demonstrate that patients who succumb to DHF do so from acute physiologic derangements caused by vascular permeability and inflammation (Bhamarapravati *et al.*, 1967; Sahaphong *et al.*, 1980).

IV. Pathophysiology of Dengue Hemorrhagic Fever

DHF cases were initially described in children living in dengue-endemic areas (Southeast Asia) with a recent dramatic increase in DHF in the Americas (Gubler and Trent, 1993; Halstead *et al.*, 1969b; Nimmannitya *et al.*, 1969). The pathogenesis and pathophysiology of severe dengue virus infections (DHF) remains incompletely understood. Early theories were based on clinical observations and seroepidemiologic studies (Halstead, 1969, 1970; Russell, 1970; Sangkawibha *et al.*, 1984). More recent work has focused on cellular immune responses to dengue virus (Green *et al.*, 1999, 2000; Kurane and Ennis, 1992, 1994; Kurane *et al.*, 1990) and the role of virus subtypes (genotypes). Age, gender, nutrition, and race may also play a role to determine the clinical severity of disease.

A. Immune System

Serologically, dengue virus infections can be classified as primary, secondary, or indeterminate. In a primary dengue virus infection, there is no preexisting anti-flavivirus antibody and the acute antibody response is marked by a large molar fraction of anti-dengue IgM antibody compared to IgG antibody (measured most commonly by antibody capture enzyme-linked immunoassay) (Innis *et al.*, 1989) with modest increases in hemagglutinating antibodies (Clarke and Casals, 1958) and neutralizing antibodies (Russell *et al.*, 1967a). A secondary antibody response pattern follows a previous infection with one or more flaviviruses and is characterized by high-level anamnestic antibody responses (Anonymous, 1997; Vaughn *et al.*, 1997). Diagnostic levels of antibody may not develop until 3 days following defervescence; a specimen collected a week following the onset of illness or 3 days following defervescence is required to rule out a dengue virus

infection. Most DHF cases occur in persons sustaining secondary dengue virus infections with heterologous dengue virus serotypes (Halstead *et al.*, 1969a). The immune enhancement theory of dengue pathogenesis was propagated by Scott Halstead to explain this seroepidemiologic observation. The immune enhancement theory states that cross-reactive nonneutralizing antibodies from a previous heterologous dengue virus infection facilitate dengue virus entry into Fc receptor–bearing cells such as monocytes and macrophages. This increase in the number of cells infected leads to more severe disease (Halstead *et al.*, 1973). While a dengue virus infection following any previous flavivirus infection may result in a secondary antibody response pattern, limited evidence suggests that dengue primary virus infection followed by a heterotypic dengue virus infection places individuals at the greatest risk of severe diseases. This assertion is based on the absence of an association between prior Japanese encephalitis virus infection or vaccination and severe dengue disease (Libraty *et al.*, 2000) and the lack of DHF cases among yellow fever–vaccinated US service members in Vietnam (Deller *et al.*, 1967). The sequence of infecting dengue virus serotypes may be significant with respect to the risk of developing DHF (Guzman *et al.*, 1990; Morens, 1994), although clearly all four dengue virus serotypes can cause DHF (Vaughn *et al.*, 1997).

The epidemiologic evidence for the immune enhancement theory of DHF is compelling. Here we discuss observations from endemic and epidemic dengue virus transmission in Thailand and Cuba.

Early seroepidemiologic studies conducted throughout Thailand demonstrated that increased severity of disease was associated with secondary dengue virus infections (Russell *et al.*, 1968). In January 1980, the prevalence of neutralizing antibody to the four dengue serotypes was estimated from random sampling of 3185 children in the municipal area of Rayong, Thailand, and contiguous suburban villages. During a dengue epidemic, which occurred later that year, there were 22 cases of virologically and clinically confirmed DSS all among children 15 years of age or younger. Serum from all 22 children with DSS revealed secondary antibody responses. Despite the high rate of DEN-1 infections in 1980, only DEN-2 viruses were recovered from DSS cases, including two DEN-1 immune children with pre-illness serum specimens. Although the pre-epidemic prevalence of antibodies to DEN-1 was the lowest to any type, children with this immunologic background contributed disproportionately to DSS cases (Sangkawibha *et al.*, 1984).

A prospective study among 4- to 16-year-old students in Bangkok pre- and post-dengue season (1980–1981) revealed that none of 47

primary dengue virus infections required hospitalization, whereas seven of 56 children with secondary infections did require hospitalization ($P = .012$). Preexistent partial dengue immunity, as determined by conventional serologic techniques, was a significant (odds ratio ≥ 6.5) risk factor for the development of DHF (Burke *et al.*, 1988).

Further evidence for the immune enhancement theory comes from the overrepresentation of infants in the first year of life on the dengue wards of the Queen Sirikit Institute of Child Health (formerly the Bangkok Children's Hospital) compared to older infants and toddlers (Nisalak *et al.*, 2003). The presence of maternal dengue antibody appears to play a role to increase the risk of DHF. In a pivotal study, dengue antibody titers in the mothers of infants with DHF correlated with the infant's age at the time of onset of severe illness. That is, the higher the level of passively acquired antibody at the time of birth, the greater the delay in the onset of disease reflecting the waning of maternal antibody from protective levels to enhancing levels (Kliks *et al.*, 1988). In addition, sera from the mothers of infants with DHF enhanced DEN-2 infections to a greater degree than did sera from the mothers of infants with pyrexia of unknown origin and toddlers with DHF; however, this work has proven difficult to replicate.

In 1975, Cuba conducted a nationwide, random and age-stratified serosurvey to detect the presence of dengue virus circulation. Only 2.6% of the Cuban population ($N = 2000$ tested) had antibodies against DEN-2 (Kouri *et al.*, 1983; Kouri *et al.*, 1989). Within 6 years, two significant dengue epidemics occurred in Cuba, one in 1977 (DEN-1) and the other in 1981 (DEN-2) (Guzman *et al.*, 1990).

The 1977 epidemic was clinically mild and affected approximately 500,000 individuals (Guzman *et al.*, 1991). A serosurvey conducted in 1978 demonstrated that 44.5% of the urban Cuban population had been infected by DEN-1 (Kouri *et al.*, 1989).

The 1981 epidemic began in May in three municipalities of Cuba (eastern, central, western) and extended across the remainder of the country within a few days. A total of 344,203 cases were reported, 10,312 of which were severe (WHO grade II-IV), and there were 158 fatalities (101 children, 57 adults) (Pinheiro and Corber, 1997). An *Aedes aegypti* vector eradication campaign was initiated and the epidemic was controlled within 4 months; the last case was reported on 10 October 1981 (Kouri *et al.*, 1989).

Kouri *et al.* identified individual clinical risk factors for severe disease or mortality from dengue. Individuals experiencing secondary dengue infections were at significantly higher risk for developing DHF. Of the children and adults with severe disease who were sampled,

pre-existing antibodies to dengue virus were discovered in 98%. The fact that there were no severe or fatal cases among 1 to 2 year olds, a cohort born after the 1977 DEN-1 outbreak and, therefore, without DEN-1 antibodies, provides further support for the hypothesis that pre-existing dengue antibodies increased the likelihood of severe disease during a DEN-2 outbreak (Kouri *et al.*, 1986).

After more than 15 years without dengue activity, Santiago de Cuba suffered a DEN-2 epidemic in 1997. There were 3012 serologically confirmed cases, 205 classified as DHF, and 12 deaths. All clinical cases occurred in adults (>15 years of age). All fatalities were classified as DHF, and all were secondary infections (Guzman *et al.*, 1999a). All but three of the 205 DHF cases were secondary infections (Guzman *et al.*, 2000b). The infection rate was estimated at 4.3% and only 3% of 13,116 estimated primary DEN-2 infections were clinically overt (Guzman *et al.*, 2000b).

Seroepidemiologic and clinical observations such as those made in Cuba in 1997 (Guzman *et al.*, 2000a) suggest differences between dengue virus serotypes in terms of their ability to cause clinically apparent disease in flavivirus naïve hosts (Barnes and Rosen, 1974; Gubler *et al.*, 1978; Maguire *et al.*, 1974; Vaughn, 2000; Wu, 1986). The previous data suggest to a great extent that the DEN-2 that affected Santiago de Cuba in 1997 was unable to cause disease outside of the context of a previous dengue virus infection. The ability of certain strains of DEN-2 and DEN-4 to cause illness in flavivirus naïve individuals may be diminished compared to other serotypes (Vaughn, 2000).

Vaughn *et al*. collected serial plasma samples from 168 children with acute dengue from two hospitals in Thailand. While they confirmed a correlation between disease severity and secondary dengue virus infection, they also demonstrated a correlation between dengue disease severity and viremia titer for DEN-1 and DEN-2 (Vaughn *et al.*, 2000). While these data provide direct support for the immune enhancement theory of dengue pathogenesis (enhanced infection of Fc receptor–bearing cells resulting in increased viral burden as reflected in viremia titers), they also support the contention that certain virus serotype subtypes or genotypes may replicate more efficiently, resulting in higher viremia levels and increased disease severity.

B. Viral Genotype

Genetic analyses of all four dengue virus serotypes have been performed using several molecular techniques to categorize dengue virus serotypes into distinct molecular groups (genotypes). Differences in

published analyses may relate to the portion of the genome being studied and the particular strains used. The genetic diversity and phylogenetic relationships of dengue virus strains isolated from different parts of the world suggest the existence of three to five DEN-1 genotypes (Chu *et al.*, 1989; Chungue *et al.*, 1995; Goncalvez *et al.*, 2002), five to six DEN-2 genotypes (Deubel *et al.*, 1993; Monath *et al.*, 1986), four DEN-3 genotypes (Chow *et al.*, 1994; Chungue *et al.*, 1993; Lanciotti *et al.*, 1994; Usuku *et al.*, 2001), and two DEN-4 genotypes (Henchal *et al.*, 1986; Lanciotti *et al.*, 1997).

It has been proposed that this genetic diversity has phenotypic implications, such as the emergence of viruses with altered antigenicity, virulence, or tissue tropism (Twiddy *et al.*, 2003). Observations of dengue epidemics in Peru during the 1990s provide an epidemiologic link to this theory.

In contrast to the Cuban and Thai experience described previously, the sequential circulation of DEN-1 followed by DEN-2 in Peru failed to produce epidemic DHF. Peru's first laboratory confirmed indigenous transmission of dengue occurred during a 1990 epidemic in Iquitos and surroundings areas (Anonymous, 1991). DEN-1 was the predominant serotype isolated from patients and *Aedes aegypti* mosquitoes and DEN-4 was isolated from a few cases (Hayes *et al.*, 1996). Although there were minor hemorrhagic manifestations (gingival bleeding) in 6.5% of patients, there were no cases of DSS. Estimates are that between 76,000 (Anonymous, 1991) and 150,000 people were infected (Phillips *et al.*, 1992). In 1995, Iquitos experienced a high transmission rate of DEN-2 (86%). However, secondary infections with DEN-2 resulted in clinically mild disease and not epidemic DHF (Watts *et al.*, 1999).

Virus sequencing of the DEN-2 strain isolated in Peru revealed differences in the 5′ non-translated region (NTR) and the 3′ NTR compared to the genotype of DEN-2 imported from Southeast Asia to other parts of the Americas (Leitmeyer *et al.*, 1999). For example, epidemics of severe dengue infections in Brazil throughout the 1990s (Nogueira *et al.*, 1993) were related to the circulation of the DEN-2 Asian genotype. In fact, the direction and distribution of the DEN-2 Asian genotype transmission from Southeast Asia to the Americas was clearly demonstrated through the identification of a common progenitor of DEN-2 viruses isolated in Brazil, Colombia, Mexico, and Venezuela with those from Southeast Asia (Rico-Hesse *et al.*, 1997). These observations support the contention that DEN-2 originating from Asia in the context of a secondary infection possess a virulence determinant that is absent from viruses originating elsewhere (White, 1999).

Another possible explanation for the mild disease observed in Peru is that pre-existing antibodies to DEN-1 in Peru were able to more effectively neutralize the American DEN-2 virus genotype (Kochel *et al.*, 2002) or enhanced virus replication to a lesser degree than that seen in Cuba. Genetic differences in the affected populations offers another possible explanation.

C. Race, Gender, Age, and Health Status

The absence of published laboratory confirmed cases of DHF in Africa despite the circulation of multiple dengue virus serotypes suggests that race may play a role in dengue pathophysiology (Gubler *et al.*, 1986). Race as a risk factor for severe dengue disease was identified following the Cuban epidemic in 1981 (Bravo *et al.*, 1987). While whites and blacks were infected at equal rates, whites were hospitalized for DHF 3.5 times more frequently than blacks with rates for those of mixed race (mulattoes) in between (Bravo *et al.*, 1987; Innis, 1995). In Haiti from 1994 to 1996, there was a significant number of dengue cases among US forces while dengue was largely unrecognized in the local population (DEN-1, DEN-2, and DEN-4 were isolated from US personnel including the DEN-2 genotype associated with severe disease in Southeast Asia) (Ehrenkranz *et al.*, 1971; Gambel *et al.*, 1999a; Trofa *et al.*, 1997). A serosurvey of children (6–13 years old) resident in Carrefour Borough, Port-au-Prince, Haiti, demonstrated that 85% had antibodies to two or more dengue virus serotypes. Despite the circulation of multiple dengue virus serotypes, DHF cases were not recorded by experienced Port-au-Prince pediatricians, further suggesting the presence of a dengue-resistance gene in black populations (Halstead *et al.*, 2001).

The rate of infection does not appear to have a strong gender bias, given that surveys of virus transmission show neither males nor females predominating (Kaplan *et al.*, 1983; Rudnick, 1986; Strickman *et al.*, 2000). Although gender may not impact the rates of symptomatic infection, females appear to disproportionately suffer from more severe disease. Halstead found this in children older than 3 years of age with secondary antibody response patterns (Halstead *et al.*, 1970). A DHF epidemic in Delhi (1996) also demonstrated girls suffering from more severe illness (Kabra *et al.*, 1999). During the 1997 Cuban epidemic, women were more frequently affected by severe disease than men (Guzman *et al.*, 1999a). A study in Malaysia demonstrated the reported incidence of DHF was higher in males. However, females suffered a higher case fatality rate (Shekhar and Huat, 1992a). This is a reversal of the more common observation that males have a poorer

prognosis for infectious diseases and may relate to the critical role of the immune response in dengue pathogenesis.

Concerning age, the dogma had been that while adults are less likely to experience DHF, they are more likely than children to become symptomatic after infection with a dengue virus, consistent with other viral infections such as hepatitis A, hepatitis B, and polio (Vaughn, 2000). The dogma was based on early reports of clinical attack rates of 75–100% among adults (McCoy and Sabin, 1946; Reed *et al.*, 1977; Simmons, 1931) versus largely subclinical infections among children (Burke *et al.*, 1988). This dogma must be called into question. For example, in the 1997 DEN-2 outbreak in Santiago De Cuba, all clinical cases occurred in adults (Guzman *et al.*, 2000c). Age-specific DHF hospitalization and death rates based on secondary DEN-2 infections were calculated from data collected during the 1981 DHF outbreak in Cuba. DHF case-fatality and hospitalization rates were highest in young infants and the elderly (Guzman *et al.*, 2002). Investigators in Bangkok found the case-fatality rate for infants with DHF to be 3.5 times higher than for older children and speculate that decreased physiologic reserves in the face of plasma leakage may explain this difference (Nisalak *et al.*, 2003). Specific commentary on age and its impact on disease severity is difficult due to changing patterns of herd immunity.

Chronic diseases such as asthma, sickle cell anemia, and diabetes mellitus appear to increase the frequency of severe disease and mortality from dengue virus infections (Kouri *et al.*, 1989). On the other hand, malnutrition has been suggested to have a protective effect, again due to the apparent requirement for a robust cellular immune response to dengue virus to experience severe disease (Sugiyanto *et al.*, 1983; Thisyakorn and Nimmannitya, 1993).

V. Geographical Distribution

Dengue virus infections have been documented in more than 100 countries (Anonymous, 1997) (Fig. 3). This section describes the historical and more recent global epidemiologic trends in the transmission of dengue virus and occurrence of DF and DHF.

A. Americas

The Americas have experienced a steady increase in dengue incidence since the 1960s (Gubler, 1987). The resurgence of *Aedes aegypti* mosquitoes following the cessation of regional control campaigns and

FIG 3. Distribution of dengue fever (*yellow*) and dengue fever and dengue hemorrhagic fever (*red*). (See Color Insert.)

the introduction of different dengue virus serotypes (DEN-1, -2, -4) (Reiter, 1996) into the Caribbean, Central, and South American regions resulted in the emergence of DHF in the late 1970s and early 1980s. The introduction of specific serotypes (genotypes) of dengue viruses may also have played a role in the dramatic increase in the numbers of severe cases (Leitmeyer *et al.*, 1999).

Outbreaks of febrile illness consistent with dengue have been reported in the Americas since the 1600s (Martinique and Guadeloupe). Throughout the 1800s, cases were reported in Peru, Virgin Islands, Bahamas, Cuba, Jamaica, Venezuela, Brazil, and Mexico. Numerous US coastal areas experienced outbreaks during the mid 1800s and at the turn of the century. Small outbreaks of dengue-like illness occurred in Cuba (DEN-2) (Guzman *et al.*, 1990) and Panama (DEN-2) (Rosen, 1958) before or during World War II and in the 1950s, disease was noted in Trinidad (DEN-2 isolated in 1953) (Anderson and Downs, 1956) and Peru (1991). During the 1960s, dengue viruses were isolated from patients in Puerto Rico (DEN-3 in 1963) (Russell *et al.*, 1966), Antigua (DEN-3) (Spence *et al.*, 1969), Jamaica (DEN-3, DEN-4) (Belle *et al.*, 1980), and Hispaniola (DEN-2) (Ventura and Ehrenkranz, 1976). In the 1970s, Barbados (DEN-2) (Evans *et al.*, 1979), Curacao (DEN-2) (Weiland *et al.*, 1978), Guatemala, and Honduras (DEN-1) (Castillo-Salgado, 2000) all had outbreaks.

In the 1980s, the Americas experienced a profound increase in the severity of dengue disease. The pattern observed was similar to that of Southeast Asia during the 1950s (Gubler, 1987). The migration from Asia to the Americas of new dengue virus serotypes, and the subsequent circulation of these serotypes throughout the region, resulted in the emergence of DHF.

Cuba suffered the first major DHF outbreak in the Americas (Pinheiro and Corber, 1997). Additional dengue activity during the 1980s was reported in El Salvador, Belize (DEN-1), Aruba (DEN-1) (Pinheiro, 1989), Bolivia (DEN-1), Guatemala (Castillo-Salgado, 2000), Honduras, Mexico (DEN-1, DEN-4) (Lorono Pino *et al.*, 1993), Nicaragua (DEN-1, DEN-2) (Kouri *et al.*, 1991), and Grenada (DEN-2) (Pinheiro, 1989). The CDC reported more than 500 cases of imported dengue in the United States between 1977 and 1994 (Rigau-Perez *et al.*, 1997).

One of the first DF-like outbreaks in Venezuela occurred during the Caribbean-Gulf-Atlantic dengue pandemic beginning in 1827, although these early epidemics may have been due to chikungunya or other causes (Ehrenkranz *et al.*, 1971). Dengue-like illnesses continued in Venezuela between 1880 and 1920 (Tejera, 1964). Following a 20-year hiatus in transmission, there was a resurgence in the 1960s (Briceno Rossi, 1964) and 1970s (Pinheiro, 1997). Disease severity worsened in Venezuela as evidenced by a DHF outbreak from October 1989 until April 1990. There were over 6000 DHF cases; 73 deaths; and DEN-1, DEN-2, and DEN-4 viruses were isolated (Uzcategui *et al.*, 2001). DEN-2 was the serotype most frequently associated with fatal cases (Gubler, 1997a). Sequence data and phylogenetic comparisons of global DEN-2 isolates indicate the ancestors of the Venezuelan viruses were Asian in origin (Uzcategui *et al.*, 2001). Dengue transmission occurred annually in Venezuela following the 1989 DHF epidemic (Barrera *et al.*, 2000).

During Operation Uphold Democracy, the deployment of U.S. troops to Haiti presented a unique disease surveillance opportunity. In 1994, dengue was an unrecognized threat for U.S. forces due to few reported cases among indigenous Haitians even though dengue was known to be endemic (Ehrenkranz *et al.*, 1971). However, dengue virus accounted for half of 103 fever admissions to a U.S. combat hospital during the first 6 weeks of deployment (Trofa *et al.*, 1997). During another surveillance period (1995), of 249 patients who presented with a febrile illness, 79 (32%) had serologic evidence of recent dengue virus infection (Gambel *et al.*, 1999a). Of great interest was the observation that the attack rate during 5 months among U.S. military (1.37 per thousand, $n = 79$ cases) was approximately one fifth of the attack rate among

American civilian contractors (6.33 per thousand, $n = 38$ cases). Although impossible to make a direct causal link, one of the major differences between the two populations was that the military had access to permethrin-treated uniforms, topical repellents, and community vector control (ground-applied ultra-low volume malathion) whereas the civilians lived on the economy with no special access to personal protective measures (Gambel *et al.*, 1999b). Throughout the operation (1994–1996), 185 strains of DEN-1, -2, and -4 were recovered from febrile US and United Nations military personnel (Halstead *et al.*, 2001).

DF and DHF have been, and continue to be, a public health problem for many countries in the Americas. Significant outbreaks in the past 15 years have occurred in Puerto Rico (Cobra *et al.*, 1995; Dechant and Rigau-Perez, 1999; Morrison *et al.*, 1998; Rigau-Perez, 1997; Rigau-Perez and Clark, 1992; Rigau-Perez *et al.*, 2001), Ecuador (DEN-1), Nicaragua (DEN-2), Brazil (Schneider and Droll, 2001), Guatemala (Castillo-Salgado, 2000), French Guiana (DEN-2) (Fouque *et al.*, 1995; Reynes, 1996; Reynes *et al.*, 1994), Panama (DEN-2) (Quiroz *et al.*, 1997), Costa Rica, El Salvador (Castillo-Salgado, 2000), Nicaragua (DEN-1, DEN-3) (Guzman *et al.*, 1996, 1997), and Guadeloupe (French West Indies) (DEN-1, DEN-2) (Strobel *et al.*, 1998).

There is little evidence that transmission of dengue viruses will slow or cease in the Americas during the beginning of this century. The WHO reports continued outbreaks of severe disease in El Salvador, Honduras, and Brazil. The continued endemic circulation of multiple dengue serotypes throughout the region and ineffective vector control portends continued dengue transmission and the accompanying social and economic burden.

Brazil is a prime example of this trend. As a result of yellow fever control programs, *Aedes aegypti* was declared eradicated from Brazil in 1973 (Pinheiro and Corber, 1997). However, 3 years later, the vector reappeared and spread throughout the country. Dengue fever was reported in the northern regions of South America by the early 1980s. In 1986, Rio de Janeiro experienced a DEN-1 (Caribbean strain) epidemic and, in 1990, a DEN-2 (Jamaican strain) outbreak. DEN-3 circulation was confirmed for the first time in Brazil (Rio de Janeiro) in December 2000 and was thought to be a Sri Lankan strain. The number of cases continues to increase and severe disease has become endemic in portions of the country (Barbosa da Silva *et al.*, 2002; Das, 2002). By week 30 of 2002, the Pan American Health Organization (PAHO) had reported 711,919 DF cases and 2229 DHF cases in Brazil due to the circulation of DEN-1, -2, and -3 (Anonymous, 2002b).

B. Africa

There have been few reports of dengue outbreaks among indigenous Africans (Gubler, 1997a). However, reports of DF epidemics have increased in frequency in this region over the past 15 years (Vazeille-Falcoz *et al.*, 1999). Despite the increase in clinically apparent disease and the circulation of all four dengue virus serotypes, DHF is rarely associated with epidemics (Anonymous, 2001a).

Christie in 1881 and Hirsch in 1883 provided the first clinical descriptions of "dengue" in Africa (Fagbami *et al.*, 1977). In 1901, the *South African News Weekly Edition* reported, "Once more . . . the borough is troubled by dengue fever. Nearly every store and office in town are deprived of some of their workers" (Adler, 1973). Outbreaks of fever in 1900, 1906, 1915, and 1926 in the Seychelles are believed to have been due to dengue (Yersin *et al.*, 1999). Epidemiologic and clinical reports suggest dengue virus circulation throughout the 1920s in South Africa and Egypt (Jupp and Kemp, 1996; Kamal, 1928). DF cases were first reported on the Comoros islands in 1943 (McCarthy and Brent, 1943).

DEN-1 and DEN-2 were isolated at the Virus Research Laboratory at the University of Ibaden, Nigeria, during a fever surveillance program started in 1964 (Carey *et al.*, 1971; Moore *et al.*, 1975). Seroprevalence studies during the late 1960s and early 1970s suggested the presence of dengue in Kenya (DEN-1) (Geser *et al.*, 1970) and Egypt (DEN-1) (Darwish and Ibrahim, 1971). In the 1970s, DEN-2 was isolated in Senegal (Robin *et al.*, 1978), while DEN-1 and DEN-2 transmission persisted in Nigeria (Fagbami, 1977, 1978; Fagbami and Fabiyi, 1976; Fagbami *et al.*, 1977; Guyer, 1972).

The Seychelles experienced impressive dengue outbreaks during the late 1970s. A DEN-2 outbreak between December 1976 and September 1977 afflicted approximately 75% of the population. The estimated case-fatality rate was 28/100,000 with some deaths attributed to meningitis and encephalitic complications (Yersin *et al.*, 1999). A similar epidemic occurred in December 1978 and January 1979 (Calisher *et al.*, 1981; Metselaar *et al.*, 1980). This epidemic was followed by a DEN-2 outbreak on Reunion Island (Coulanges *et al.*, 1979; Lassalle *et al.*, 1998).

Reports of dengue virus circulation throughout Africa increased during the 1980s. DEN-2 was documented in Nigeria, Kenya, and Burkina Faso (Adekolu-John and Fagbami, 1983; Gonzalez *et al.*, 1985; Johnson *et al.*, 1982a). In 1983, DEN-2 and DEN-4 were isolated in Senegal (Saluzzo *et al.*, 1986) and infection was documented among

expatriates (DEN-2) in Mogadishu (Saleh *et al.*, 1985). Port Sudan Hospital isolated DEN-1 and DEN-2 viruses from febrile patients (Hyams *et al.*, 1986). An epidemic of dengue-like illness in Mozambique in 1984 yielded the first evidence of DEN-3 virus transmission in Africa (Gubler *et al.*, 1986). DEN-2 circulated in Somalia (Botros *et al.*, 1989) and DEN-1 had a minimal presence in Angola late in the decade (Gubler, 1997a).

In the 1990s, DEN-2 circulated in Senegal (Traore-Lamizana *et al.*, 1994; Zeller *et al.*, 1992), various locales in Kenya (Johnson *et al.*, 1982b; Johnson *et al.*, 1990), and Djibouti (Rodier *et al.*, 1995, 1996). During Operation Restore Hope, Somalia (1992–1993), DEN-2 and DEN-3 viruses were recovered from US soldiers presenting with febrile illnesses (Kanesa-thasan *et al.*, 1994; Sharp *et al.*, 1995). In April 1993, DEN-1 was isolated from sera collected from the Grande Comore Island, Anjouan, and Moheli in the Seychelles (Zeller *et al.*, 1999). DEN-1 has been isolated in Abidjan (Tolou *et al.*, 2001), South Africa (Blackburn *et al.*, 1987), and Côte d'lvoire (Akoua-Koffi *et al.*, 2001; Durand et al., 2000). Anti-dengue antibodies have been detected on the islands of Mauritius and Rodrigues (Schwarz *et al.*, 1994). Little dengue activity has been reported since 1998 (Anonymous, 2001b).

The absence of published laboratory-confirmed cases of DHF in Africa despite the circulation of multiple dengue virus serotypes remains largely unexplained (Gubler *et al.*, 1986). Limited surveillance and poor reporting may be a factor. It may be that the sequence or timing of serotype introduction has been less than optimal to induce severe disease. It could also be that dengue virus strains circulating in Africa to date have been less virulent than strains elsewhere (Watts *et al.*, 1999).

Dengue viruses appear to be transmitted in two cycles; the first involves human hosts and mosquito vectors. The second cycle is a zoonotic or sylvatic cycle in sylvatic habitats of Africa and Malaysia, involving nonhuman primate reservoir hosts and several different *Aedes* mosquitoes (Gubler, 1988; Gubler and Trent, 1993; Wang *et al.*, 2000b). In West Africa, nonhuman primate cycles have been identified in several countries. DEN-2 isolates, believed to be genetically and evolutionarily distinct, have been isolated from *Aedes* (*Stegomyia*) *africanus, Aedes* (*S.*) *leuteocephalus, Aedes* (*S.*) *opok, Aedes* (*Diceromyia*) *taylori*, and *Aedes* (*D.*) *furcifer* (Cordellier *et al.*, 1983; Rouche *et al.*, 1983). There is evidence for the existence of a sylvatic cycle of dengue virus, which is clearly distinct from human outbreak viruses (Rico-Hesse, 1990). Sequencing dengue viruses from different

geographical regions and determining areas of nucleotide homology may provide a framework to divide strains of dengue into sylvatic and human/mosquito/macaque groups (Shurtleff *et al.*, 2001). The failure to isolate sylvatic strains of dengue virus from febrile patients suggest that sylvatic strains could be naturally attenuated for humans and at least suggests the possibility that an effective dengue vaccine that stops human-mosquito-human transmission could lead to the eradication of virulent dengue (Robert Shope, personal communication).

C. Pacific Region

Dengue has been described in the Pacific region for over 100 years (Halstead, 1980). Some have speculated that fever outbreaks described in the Chinese literature dating back to the Chin Dynasty (265 to 420 AD), Tang Dynasty (610 AD), and the Northern Sung Dynasty (992 AD) were dengue (Gubler, 1998). Soldiers stationed in the Pacific theater during World War II introduced dengue viruses throughout Southeast Asia, Japan, and the Pacific Islands (Nisalak, 1997). Since World War II, dengue epidemics have become more and more frequent in the region (Fauran, 1996; Halstead, 1992; Pinheiro and Corber, 1997; Rehle, 1989). Today, dengue is one of the most important arboviral diseases of the southwest Pacific Islands (Le Gonidec and Fauran, 1981) and is reported to occur in 33 of 37 countries in the Western Pacific region (Anonymous, 2000).

Dengue was a disease of great military importance during WW II. The deployment of non-immune troops to dengue-endemic areas with unchecked vector populations resulted in large epidemics of disease and significant decreases in combat effective troop strength. Between 1942 and 1945, the highest annual attack rates were in the Southwest Pacific (32 cases/1000 troops), Central and South Pacific (21/1000), and China-Burma-India regions (18/1000). The highest yearly rate among all overseas troops occurred in 1944 (13/1000) and sharply decreased the following year (4/1000) with the implementation of effective vector control (McCoy and Sabin, 1946).

McCoy and Sabin described the U.S. Army's dengue experience. Epidemics among troops were recorded in the Northern Territory and Queensland (1942), Espíritu Santo (1943), New Caledonia (1943), New Guinea (1944), and the Philippines (1945) (McCoy and Sabin, 1946). The Japanese port cities of Nagasaki, Kure, Sasebo, Kobe, and Osaka also suffered significant disease during World War II. Reports suggest there were more than 2 million cases of dengue between 1942 and 1945 in Japan (Sabin, 1952).

An extensive dengue outbreak among U.S. forces occurred in 1944 in the Marianas Islands. The rainy season (August) in Saipan brought abundant populations of *Aedes aegypti* and *albopictus* mosquitoes. The dengue rate among Army, Navy, and Marine Corps personnel living in the barracks was 300 per 1000 per annum and rose to 3560 per 1000 per annum by September. Despite the use of DDT to control vector populations, more than 20,000 dengue cases are believed to have occurred on Saipan by late October (McCoy and Sabin, 1946).

Dengue viruses largely disappeared from the Pacific region following WW II until two small DEN-3 outbreaks occurred in Tahiti during the 1960s (Gubler, 1997a). The 1970s saw significant outbreaks of disease in Fiji (Moreau *et al.*, 1973), American Samoa (Gubler, 1997a), the Pacific Island Kingdom of Tonga (DEN-1, -2) (Gubler *et al.*, 1978), Futuna (Horne Islands) (DEN-1) (Fauran *et al.*, 1978), Malaysia (Shekhar and Huat, 1992a,b), Vanuatu (DEN-1, -2, -3, -4) (Taleo *et al.*, 2000), New Caledonia (Fauran, 1996), and Niue Island (Barnes and Rosen, 1974).

In 1980, another dengue epidemic occurred on the island of Niue and was considered a significant contributor to mortality among the small indigenous population (Taylor *et al.*, 1987). In 1983, a DHF outbreak was reported in the Philippines (Songco *et al.*, 1987) and Palau experienced outbreaks in 1988 and again in 1995 (Hansen, 2001). Dengue returned to Vanuatu in 1989 (Guillo, 1984; Perolat and Reeve, 1992).

Taiwan reported epidemics in 1902, 1915, throughout the 1920s, 1931, 1935 (Halstead, 1980), and 1942–1943 (King *et al.*, 2000). Hankow, China, suffered a dengue epidemic in September 1945, affecting approximately 80% of the population (McCoy and Sabin, 1946). Dengue virus circulation in Taiwan remained silent after the 1943 outbreak for almost 37 years until a DEN-2 epidemic struck the islet of Hsiao-Liu-Chiu in 1981, a DEN-1 epidemic in southern Taiwan in 1987, and a DHF epidemic (DEN-3) in 1994 (King *et al.*, 2000).

Throughout the 1990s, more than 1 million cases of dengue were reported in the western Pacific region. The Philippines, Malaysia, Cook Islands, French Polynesia, New Guinea, and Australia (Russell, 1998) all reported cases early in the decade. Tahiti experienced a DHF epidemic (DEN-3) in 1990. Secondary DEN-3 infections on a background of recent DEN-1, and more remote DEN-2 circulation explained the severity of disease observed (Halstead, 1997). In 1994, American Samoa reported their first cases ($N = 246$) of the 1990s and Vanuatu and New Caledonia confirmed continued dengue virus circulation. Numerous regions experienced increased transmission rates during the mid- to late 1990s to include New Caledonia (Bouree *et al.*, 2001), Brunei

Darussalam, the Cook Islands, Malaysia, French Polynesia, Micronesia, Palau, and New Zealand. Guam reported their first case of dengue in 1997 (Anonymous, 2000). French Polynesia experienced a significant outbreak beginning in 1996 with a 30% to 60% attack rate and severe illness with shock, hemorrhage, and encephalitis (Delebecque, 1997).

In 1998 and 1999, there were 356,554 and 64,066 cases of DF and DHF with 1470 and 112 deaths, respectively, in the Pacific region (Anonymous, 2001b). Fiji suffered a massive outbreak and the North Mariana Islands reported their first cases of the decade in 1998. Vanuatu experienced a dengue resurgence following three years without reported disease, and Wallis and Futuna reported 395 cases and one death (Anonymous, 2000). This century, Palau (DEN-1), Samoa, and the atolls of Tokelau (Hansen, 2001; Kiedrzynski, 2002; Region, 2002) have all reported outbreaks.

Dengue viruses continue to circulate throughout the Pacific region. The incidence of DHF may, in fact, increase despite many of the islands' geographic isolation, due to the ease of international travel and the global communities' desire for access to tropical settings.

D. South and Southeast Asia

World War II caused many ecologic and demographic disruptions in Asia and the Pacific to contribute to the evolution of the DF/DHF pandemic. Destroyed water systems and the resulting reliance on manmade water receptacles provided excellent breeding grounds for *Aedes aegyptii* mosquitoes. The transport (deployment) of viremic soldiers, mosquitoes, and mosquito eggs to new geographic regions introduced dengue viruses into previously pristine areas or new dengue virus serotypes into areas without multiple serotypes circulating (Gubler, 1997a, 1998). Seroepidemiologic studies conducted since World War II in Asia and the Americas have demonstrated that the co-circulation of different dengue virus serotypes within the same geographic region is associated with epidemics of severe disease (DHF) (Halstead *et al.*, 1967; Innis, 1995). DHF was recognized in children in Thailand and the Philippines during the 1950s (Thai and Philippine hemorrhagic fever, respectively). The expansion of DHF in Thailand was closely associated with improvements in transportation systems that led to distribution of both the vector and the virus (Wellmer, 1983). Hemorrhagic fever was first identified in South Vietnam in 1960, two major DHF outbreaks occurred shortly thereafter (1960, 1963) (Ha *et al.*, 2000; Halstead *et al.*, 1965; Mirovsky *et al.*, 1965). The incidence of DF/DHF steadily increased and is now endemic or hyperendemic throughout Southeast Asia and remains a

major cause of infant and childhood mortality (Cohen and Halstead, 1966; Nisalak, 1997; Nisalak *et al.*, 2003).

DEN-3 and DEN-4 were first isolated in the Philippines and Thailand (Hammon *et al.*, 1960). DEN-4, DEN-3, and DEN-2 viruses were isolated from hemorrhagic fever patients in Manila in 1964 (Basaca-Sevilla and Halstead, 1966a, b) and in Singapore in 1965 (Chan *et al.*, 1965). All four dengue virus serotypes would subsequently be isolated in Calcutta (Ramakrishnan, 1964), Kho Samui, Thailand (Thavara *et al.*, 1996), Indonesia (Sumarmo, 1987), and numerous other Southeast Asian locations (Balaya *et al.*, 1969; Chatterjee *et al.*, 1966; Myers *et al.*, 1965, 1968). Much was learned from case studies (Halstead *et al.*, 1963) and prospective field studies conducted in Thailand during the 1960s to include the observation that secondary antibody response patterns were associated with severe disease (Russell *et al.*, 1967b) that lead to the immune enhancement theory of dengue pathogenesis (Halstead *et al.*, 1973). Additionally, it was recognized that early, aggressive fluid management could be life-saving. However, care must be taken to avoid fluid overload (Nimmannitya, 1987). Myanmar experienced one of the first major DHF outbreaks of the 1970s (Min *et al.*, 1975a; Thaung *et al.*, 1975) and Malaysia followed soon thereafter (Lim *et al.*, 1974). Three epidemics began in south China in the late 1970s. The first (DEN-4) and second (DEN-1) took place in Guangdong Province between 1978 and 1979 and the third, an epidemic containing cases of DHF (DEN-3), occurred on Hainan Island in 1980 (Qiu *et al.*, 1993). Beginning in 1976, significant DHF outbreaks were reported in Thailand, Vietnam, Indonesia (Kho *et al.*, 1981; van Peenen *et al.*, 1978), and Myanmar (Gubler, 1988).

In the 1980s, Cambodia (Rathavuth *et al.*, 1997), Taiwan (Chen, 1989; Wu, 1986), Myanmar, Malaysia, Indonesia, and Thailand all observed increases in the incidence of DF/DHF (Chen, 1989; King *et al.*, 2000; Nisalak, 1997). In 1982, Malaysia experienced the worst DF/DHF (DEN-1, DEN-3) outbreak to that point in its history with a total of 3005 DF/DHF cases and 35 deaths (Fang *et al.*, 1984). In 1984, 24 American military personnel were hospitalized with DF at Clark Air Base, Philippines (DEN-1, DEN-2, and DEN-3) (Hayes *et al.*, 1989). Cases of DHF in Vietnam continued to increase throughout the 1980s and by 1997, Vietnam was experiencing approximately 38,000 cases per year (Nisalak, 1997). In 1987, Thailand experienced their worst dengue outbreak (DEN-3, DEN-2) in 30 years with 174,285 cases and 1007 deaths (Ungchusak and Kunasol, 1988).

Dengue reporting increased further throughout Asia and Southeast Asia during the 1990s with large outbreaks of DF/DHF occurring in

India (Padbidri *et al.*, 1995; Thakare *et al.*, 1996; Vajpayee *et al.*, 1999). In 1995, Cambodia experienced a tenfold increase in the number of dengue infections (Rathavuth *et al.*, 1997) and China reported more than 6000 cases, their highest total for the decade. That same year Laos, Malaysia, Singapore, and Vietnam all had increases in their case numbers (Anonymous, 2000).

1998 was another particularly heavy year for dengue virus transmission, not only in Asia, but globally. The WHO collected reports on 1.3 million cases of dengue and DHF with 3442 deaths. Southeast Asia reported to the WHO 218,859 dengue cases and 2075 deaths (Anonymous, 2001b). Indonesia reported 32,665 cases and 774 deaths (Anonymous, 1998). This same year, more than 16,000 dengue cases were reported in Cambodia, all severe enough to necessitate hospitalization. Vietnam reported its highest yearly total of the decade with 234,866 cases and 383 deaths (Anonymous, 2000).

In 2000, Bangladesh suffered its first reported DHF outbreak (DEN-3) marked by numerous fatal complications and reports of three co-infections (two DEN-3/-2, on DEN-3/-4) (Aziz *et al.*, 2002; Rahman *et al.*, 2002). Since the peak in 1998, the cycle of epidemics every 3 to 5 years appears to be continuing (Anonymous, 2001b). A study of dengue incidence in Bangkok over a 33-year period demonstrated cyclic peaks that centered around a 3-year periodicity not related to long-term weather patterns (El Niño, La Niña) but more likely related to population immunity (Hay *et al.*, 2000).

E. Europe, Mediterranean, and the Middle East

Clinical illnesses compatible with dengue were described in Cadiz and Seville, Spain, between 1784 and 1788. Disease occurred throughout the 1800s in the Middle East regions of Suez (1824), the Arabian coast (1835), Yemen (1870–1873), and Israel (1889–1890) (Gubler, 1997a). During WW II, there were scattered cases of dengue in European, Mediterranean, and Middle East theaters. The number of cases among U.S. Army troops in these regions between 1942 and 1945 were 91, 358, and 35, respectively (McCoy and Sabin, 1946).

In 1927 and 1928, an extensive dengue outbreak occurred in Greece. Estimates place the total number of infections at greater than one million (Sabin, 1959). Mild disease occurred early in the epidemic, but by August of 1928 outbreaks of severe illness began in Athens and Piraeus. Of 650,000 reported cases there were 1061 deaths (Halstead and Papaevangelou, 1980). Retrospective serologic investigations clearly implicate DEN-1 as one of the circulating serotypes (Theiler

et al., 1960). Additional studies demonstrate the likely possibility DEN-2 also circulated. Sequential infections ending with DEN-2 may explain the trend toward severe disease later in the epidemic (Halstead and Papaevangelou, 1980).

Travelers and expatriates import numerous dengue cases into Europe and the Mediterranean every year. Most infections are acquired in the hyperendemic areas of Southeast Asia or Latin America (Janisch *et al.*, 1997). Illnesses resulting from infection usually result in DF; however, cases of DHF have been reported (David *et al.*, 2000; Ligtenberg *et al.*, 1991; Lopez-Velez *et al.*, 1996). Britain (Jacobs *et al.*, 1991), Germany (Eisenhut *et al.*, 1999; Schmitz *et al.*, 1996), the Czech Republic (Chalupa *et al.*, 2001), France (Chippaux and Poveda, 1993), Israel (Schwartz *et al.*, 1996), the Netherlands (Ligtenberg *et al.*, 1991), Norway (Jensenius *et al.*, 1997), Spain (Gascon *et al.*, 1998), and Sweden (Niklasson and Vene, 1996; Wittesjo *et al.*, 1993) have all reported cases in returning travelers and expatriates.

Global climatic changes, the importation of tires or other *Aedes* breeding containers, and the subsequent migration of dengue vector populations increase the possibility of indigenous dengue transmission in European, Mediterranean, and Middle East regions (Knudsen *et al.*, 1996; Rodhain, 1995, 1996; Shope, 1991; Ward and Burgess, 1993).

VI. Ecology of Dengue from a Biological Perspective

Arthropod-borne pathogens can be divided into two broad categories: those which depend on zoonotic cycles for survival and those which depend on human cycles. Among the familiar examples of zoonotic pathogens that affect humans are *Orientia tsutsugamushi* (scrub typhus disease) and West Nile virus. Dengue virus and *Plasmodium* spp. (malaria) are examples of arthropod-borne pathogens that depend on humans for basic maintenance. The contrasts in management, vector associations, and geographic distribution of zoonotic and human arthropod-borne pathogens provide a framework for considering the ecology of dengue virus.

Zoonotic pathogens are generally managed by reducing human contact with the vector. Because a cycle of transmission exists outside of the human community, breaking the cycle and eradicating the pathogen from a region is usually not practical unless the primary animal reservoir is also managed (e.g., pigs and Japanese encephalitis virus) (Nathin *et al.*, 1988). Reduction of the impact of these diseases on public health depends on suppressing vector populations near

humans, reducing human contact with vectors by modifying the environment or human distribution, separating the animal reservoir of the pathogen from concentrations of human population, or application of interventions directed at the individual (vaccination, chemoprophylaxis, or personal protective measures). The zoonotic pathogen cycles are typically immobile and restricted geographically because they depend on the presence of particular ecologic associations of the vectors and animal reservoirs. A notable exception to this pattern is West Nile virus, which has spread very rapidly into new zoonotic cycles (both animal reservoirs and vectors) in North America (Johnson and Irani, 2002).

Although fewer in number, the impact of human-associated arthropod-borne pathogens appears to be more severe. The ravages of epidemic typhus (*Rickettsia prowazekii*) (Scoville, 1948), malaria (principally *Plasmodium vivax* and *falciparum*) (Mouchet *et al.*, 1998; Roberts *et al.*, 1997), urban yellow fever (Tomori, 1999), and pneumonic plague (*Yersinia pestis*) (Butler, 1989) are notable as major determinants of demographics and history. While sylvatic transmission of dengue viruses does occur (see previous section), the current pandemic of dengue justifies adding this virus to the list of human-associated arthropod-borne pathogens that have changed the way people live.

In contrast to the zoonotic pathogens, management of the human-associated pathogens can lead to regional eradication. Epidemic typhus is now controlled throughout most of the world, with only occasional major outbreaks (Raoult *et al.*, 1997). Malaria remains one of the major public health problems, but is effectively managed in much of its former range (Faust, 1949). Yellow fever is still a threat, but strategies combining vector control and systematic vaccination have stopped or prevented new urban epidemics (Adu *et al.*, 1993). Plague continues to circulate in wild animals, but recent urban outbreaks have been curtailed by quarantine, rat control, and flea control (Butler, 1989). Possibly because it is only occasionally fatal, management of dengue through vector control and quarantine is haphazard at best. As documented previously, transmission of this virus continues with little or no control.

The distribution of human-associated pathogens obviously follows the distribution of humans. Extensive travel during the late nineteenth and twentieth centuries removed any barriers that remained to maximal distribution of any of these pathogens. The only geographic limits on the human-associated vector-borne pathogens relate to environmental requirements of the vectors and of the pathogen in the vectors. Dengue provides one of the clearest examples of those kinds

of environmental requirements, because most dengue transmission depends on a single vector species, *Aedes aegypti*.

Aedes aegypti is arguably the most human associated of all mosquitoes (Kittayapong and Strickman, 1993). Outside of its native range in Africa, it rarely occurs in natural sites (Chadee *et al.*, 1998; Kittayapong and Strickman, 1993). Several aspects of the species predispose it toward life with humans. First, the eggs are resistant to drying and can remain in a quiescent state for months. As a result, eggs deposited on items commonly transported by people (e.g., tires, water containers) have a good chance of survival during phoretic movement. Eggs deposited above the water line hatch when flooded by changes in water level, so that normal household activity is likely to create hatches of larvae more frequently than would occur from natural rainfall. Females prefer to oviposit in relatively clean water (compared to fetid water, like that associated with fecal accumulations), as is often found in homes where there is no running water. They are capable of finding small crevices to enter a container, so that a cover must be well-sealed to protect water from oviposition (Strickman and Kittayapong, 1993). Although outdoor sites are colonized, there is a strong tendency to use habitats located under a roof or indoors. Larvae develop fast, 6 days at conducive temperatures with adequate nutrition) (Christophers, 1960) and have the behavior of staying at the bottom when disturbed (Strickman, 1989). As a result, cleaning containers must be frequent and partially emptying a container is unlikely to kill a large proportion of larvae. Adult mosquitoes do not travel great distances (usually less than 200 m) (Ordonez-Gonzalez *et al.*, 2001), reducing losses from emigration. The females usually have a strong preference (>90%) for feeding on humans (Scott *et al.*, 1993), even in the presence of other animals. Strong selection for host preference is probably re-enforced by the unusual behavioral pattern of males, which are also attracted by the host (unpublished data from landing collections in Thailand). This behavior may facilitate formation of mating pairs.

The blood-feeding habits of *A. aegypti* have come under closer scrutiny in recent years, adding details to some aspects of its vectorial capacity. Under some conditions, and perhaps the majority of the time, females are unusual among mosquitoes in avoiding sugar as an energy source (Edman *et al.*, 1992). Even though females in the laboratory readily take sugar meals at any time during their lives, samples of wild-caught females in Thailand and Puerto Rico rarely contain sugar. As a result, females take more frequent blood meals, using the nutrients for energy as well as for egg production. This association is

apparently related to the preference for human blood. Low in one of the essential amino acids for egg production (isoleucine), human blood results in fewer eggs produced compared to other blood sources. In the laboratory, the net effect is greater fecundity over the life of the mosquito if it avoids sugar meals (Scott *et al.*, 1997). The combination of the preference for human blood and the need to feed frequently contribute toward the power of *A. aegypti* as a vector of human-associated pathogens. Although poorly documented, there is an indication that *A. aegypti* in the United States preferred dogs over humans as a blood source (Edman *et al.*, 1998). Now that *A. albopictus* has replaced *A. aegypti* in much of its former range in the United States (Lounibos *et al.*, 2002), it would be difficult to study this problem thoroughly. Considering the numerous colonies adapted to rodent feeding in the laboratory, *A. aegypti* readily changes host preference when humans are not available.

One of the challenges in the life cycle of *A. aegypti* is larval nutrition. The species is able to tolerate a wide variety of nutrition levels by producing larger or smaller adults. A wild population of the species generally includes a range of adult sizes, reflecting the nutrition available in the many containers contributing developmental sites (Strickman and Kittayapong, 2003). Size affects the vectorial capacity of the mosquito, though arguments exist for either small or large individuals being more capable vectors. On the one hand, smaller individuals probe and feed more often than larger individuals (Naksathit *et al.*, 1999; Scott *et al.*, 2000). On the other hand, larger individuals are physiologically more competent (Sumanochitrapon *et al.*, 1998) and longer lived (unpublished data from landing collections in Thailand). In an unpublished study of dengue transmission in rural Thailand, one author (Strickman) found a significant correlation between the size of wild-caught *A. aegypti* and areas of a village where dengue transmission was most intense. Given the high mortality rate of *A. aegypti* in the field (Day *et al.*, 1994; Harrington *et al.*, 2001), increased longevity is probably a distinct multiplier of the chance of an individual mosquito living the 7–12 days necessary for the virus to develop to infectious levels at typical temperatures (30–34 °C) (Watts *et al.*, 1987).

Individual populations of *A. aegypti* vary in physiologic susceptibility to dengue virus (Sumanochitrapon *et al.*, 1998). The adaptive significance of this variation is not clear because the effect of viral infection on the mosquito appears to be small (Platt *et al.*, 1997). It is possible that the variation of susceptibility is not the expression of a specific immune mechanism, but instead the coincident result of other physiologic factors like concentration of trypsin in the digestive

system. From a practical standpoint, variation in susceptibility might change the estimated level of vector control required to stop dengue transmission.

One of the salient features of the relationship of dengue virus, *A. aegypti*, and humans is tremendous historical change. The distribution of the vector has expanded on a large scale in recent centuries and the distribution on a smaller, country scale has changed in recent decades (Gubler, 1997b). For the most part, dengue virus transmission has followed the distribution of *A. aegypti*. This has limited the distribution of dengue to tropical and subtropical climates that are conducive to survival of the vector. In addition, the extrinsic incubation period of the virus (time necessary in the mosquito to replicate to infectious levels) is highly dependent on temperature (Watts *et al.*, 1987). Up to the point when survival of the vector is threatened, higher temperatures should result in shorter extrinsic incubation periods and a greater likelihood that individual mosquitoes will live long enough to transmit the virus. A very useful model of this relationship has been published (Focks *et al.*, 2000) with the output of number of mosquitoes per person necessary to maintain an outbreak of dengue transmission. The model estimates mosquito longevity and extrinsic incubation period based on temperature and uses estimates of herd immunity. The source of temperature data has a great influence on the model outcome, pointing out the need for research on actual temperatures to which mosquitoes are exposed.

VII. Control of DF/DHF by Management of the Mosquito Vector

Until a dengue vaccine is commercially available for mass immunization of populations residing in endemic regions, control of *Aedes* mosquitoes in the subgenus *Stegomyia* remains the only means of prevention and control of DF/DHF (Goh, 1997). There are examples of successful vector control, such as the Americas' campaign to control yellow fever during the 1950s. However, by 1997 with the exception of Canada, Chile, and Bermuda, all countries in the Americas were reinfested (Pinheiro and Corber, 1997). There are many possible reasons for the expansion of the distribution of the anthropophilic dengue vectors, *A. aegypti* and *albopictus*. Rural-urban migration has resulted in overcrowded cities with housing and sanitation deficiencies. This has led to an increase in breeding sites such as reservoirs for drinking water and disposable containers, which accumulate water (used cans, plastic and glass bottles, tires) (Hwang and Roam, 1994; Tauil, 2001).

Modern transportation systems have contributed to the spread of vectors (Wellmer, 1983).

In general, vector control may be accomplished by long-term modification of the environment, temporary or seasonal manipulation of the environment, and modifying or changing human lifestyles and practices to reduce contact with infective vectors (Ault, 1994).

Successful control of *A. aegypti* has been achieved by applying the standard steps of integrated pest management: Surveillance, treatment, and monitoring (Rose, 2001; Wang, 1994; Wang *et al.*, 2000a). Surveillance schemes have most often emphasized the larval stage because the larvae are easily found in household containers (Gubler, 1998; Ishak *et al.*, 1997). Various schemes for operational surveillance of larvae have been used (Rawlins *et al.*, 1998), including those provided as the standards by the World Health Organization (Anonymous, 1986). Recent work has shown the value of complete counts of pupae, eliminating the uncertainty of larval survival from surveillance (Focks and Chadee, 1997). Complete counts of pupae can be translated accurately into measures of the burden of female mosquitoes on the local population, arriving at a highly relevant measure of risk and thresholds for viral transmission (Focks and Chadee, 1997; Focks *et al.*, 2000). Direct counts of adult mosquitoes would be more useful if they were easier to perform. Landing collections are useful but time consuming, and they inherently involve the risk of viral transmission (Anonymous, 1986). The only mechanical means of collecting adults currently proven and available is aspiration with a large-bore device applied to interior walls of infested homes. An indirect method of sampling adults is to place artificial oviposition sites (essentially a black cup with water) in homes, collecting and counting the eggs deposited (Yap, 1975). These ovitraps provide a sensitive means to detect the presence of the species, but they can be a misleading indicator of the number of adults in the area (Fay and Eliason, 1966; Fay and Perry, 1965). The number of eggs is particularly problematic during an effective vector control campaign because the successive removal of larval sites makes the ovitraps increasingly attractive to remaining mosquitoes. Any successful surveillance program enables operational entomologists to prioritize treatment efforts.

Treatment of larval *A. aegypti* can be easy once the larval sites are identified. A number of effective and safe insecticides are available for larval treatment—chiefly the organophosphate temephos, the insect growth regulator methoprene (Dorta *et al.*, 1993), and the protein-based toxin of *Bacillus thuringiensis* israelensis (Kroeger *et al.*, 1995). Cost and labor can be the principal limitations on successful larval

control efforts. Various large programs to enlist the help of the public have met with only partial success, although any program can expect to achieve significant reduction in the mosquito populations simply through education (Espinoza-Gomez *et al.*, 2002; Lloyd *et al.*, 1992). Costs of insecticide can be reduced to a very low level by using alternative methods of larval control, including elimination of larval sites, use of covers, use of predators like fish and copepods, and careful regular cleaning of household containers (Nam *et al.*, 2000; Vu *et al.*, 1998).

Unfortunately, the methods for killing adult *A. aegypti* are much less successful than those for killing larvae. Fogging with ultra-low volume insecticides applied from the street outside of homes appears to be ineffective, probably because the insecticide does not impact mosquitoes located indoors (Fox and Specht, 1988; Newton and Reiter, 1992; Perich *et al.*, 1992, 2000). Fogging indoors with thermally or mechanically generated aerosols can probably be useful if applied over a wide area (Osaka *et al.*, 1999), but it is seldom used in more than a few houses surrounding known human cases of dengue. Although targeting such treatments to human dengue cases is attractive in concept, the strategy probably does not work in practice because of the large number of asymptomatic or low-symptomatic viremic people. There are only limited registered insecticides for indoor application to surfaces and their use for *A. aegypti* have not been studied well. Application of residuals to potential oviposition sites was useful during eradication campaigns in the Americas, when DDT was the principal toxicant (Anonymous, 1986). Use of this method with modern insecticides has only recently been explored. Regular use of household aerosol insecticides might also make an impact, but more work would be needed to prove this technique. Any successful technique for adult *A. aegypti* control would be a welcome addition, especially if it targeted older females most likely to transmit the virus.

Monitoring the success and maintenance of vector control programs is seldom applied with the vigor required for sustained interruption of viral transmission. The techniques could be essentially the same as those for surveillance, but they need to be applied in the apparent absence of the successfully controlled vector. It could be argued that the general lack of investment in the monitoring portion of integrated pest management has been a common cause of failure of dengue vector control programs.

Personal control efforts include the avoidance of the day-biting vectors of dengue through the use of screens, household insecticide

products, long pants and long sleeve shirts, and the consistent use of mosquito repellents containing at least 30% DEET (Yap *et al.*, 1994).

VIII. Future Prospects

Dengue vaccine development efforts date back more than 70 years to attempts to prevent virus transmission using infectious human plasma treated with ox bile or virus grown in live mosquitoes and inactivated with formalin (Simmons *et al.*, 1931). Sabin and Schlesinger undertook the first attempts to immunize using mouse-passaged live-attenuated dengue viruses (Sabin, 1952; Schlesinger *et al.*, 1956). Dengue viruses were subsequently attenuated for man by passage in primary dog kidney (PDK) cell culture (Bhamarapravati and Yoksan, 1989, 2000; Bhamarapravati *et al.*, 1987; Halstead *et al.*, 1984; Vaughn *et al.*, 1996).

Development of dengue vaccines has been hindered by: 1) the need for vaccines that simultaneously protect against the four types of dengue virus; 2) concerns about "immune enhancement" leading to more serious disease following the waning of vaccine-elicited immunity; 3) poor animal disease models for vaccine testing; and 4) the lack of *in vitro* markers of attenuation for humans.

Numerous monovalent DEN-1 (McKee *et al.*, 1987; Sabin, 1952; Wisseman *et al.*, 1963), DEN-2 (Bancroft *et al.*, 1984; Eckels *et al.*, 1976, 1980; Harrison *et al.*, 1977; Schlesinger *et al.*, 1956), DEN-3 (Innis *et al.*, 1988), and DEN-4 (Eckels *et al.*, 1984; Hoke *et al.*, 1990; Marchette *et al.*, 1990) vaccine candidates have been developed and clinically tested. Tetravalent dengue vaccine candidates are now in phase 2 clinical testing (Bhamarapravati and Yoksan, 2000; Kanesathasan *et al.*, 2001; Sabchareon *et al.*, 2002; Sun, 1999). Although promising, success following this traditional approach to viral vaccine development is not assured. Issues include reactogenicity in the form of mild dengue-like symptoms, inconsistent tetravalent immune responses due to replicative interference between vaccine virus serotypes, and the risk of enhanced reactogenicity when the vaccines are given to individuals partially immune to Flaviviruses.

Several second-generation dengue vaccines have been created using molecular approaches that hope to circumvent the uncertainties of traditional trial-and-error approaches to virus attenuation (Barrett, 2001). Full-length infectious clones of wild-type dengue viruses have been created and selectively mutated to produce vaccine candidates with biologically plausible explanations for attenuation and other

characteristics important to commercial vaccine production (Durbin *et al.*, 2001; Hanley *et al.*, 2002; Markoff *et al.*, 2002; Whitehead *et al.*, 2003). A related approach is to use clones of dengue virus or other flaviviruses (yellow fever virus) with clinically proven evidence of attenuation as molecular virus backbones and to insert dengue virus structural genes to make chimeric virus vaccines that should provide tetravalent protection (Guirakhoo *et al.*, 2002; Huang *et al.*, 2000; Lai *et al.*, 1998; Monath *et al.*, 2002). Inactivated whole virus and recombinant subunit vaccines are being pursued (Putnak *et al.*, 1999; Simmons *et al.*, 2001a). DNA vaccines also show promise as a standalone approach or as a "prime-boost" with inactivated vaccines (Chang *et al.*, 2001; Putnak *et al.*, 2003; Raviprakash *et al.*, 2001; Simmons *et al.*, 2001b).

The current preclinical standard to evaluate candidate vaccine efficacy is to prevent viremia in rhesus macaques when the animals are challenged with wild-type dengue virus following vaccination (Eckels *et al.*, submitted). However, this is not a disease model. Recent attempts to reintroduce a human challenge model offers an approach to evaluate the efficacy of modern vaccines before field efficacy testing in large numbers of susceptible volunteers (W. Sun, personal communication). Experimental infection of human volunteers with dengue virus has been produced in many hundreds of volunteers since the beginning of the last century without untoward effects (Ashburn and Craig, 1907; Cleland *et al.*, 1919; Eckels *et al.*, 1984; Graham, 1902; Hotta, 1952; Innis *et al.*, 1988; McKee *et al.*, 1987; Misao, 1944; Sabin and Schlesinger, 1945; Sawada *et al.*, 1943; Schlesinger *et al.*, 1956; Siler *et al.*, 1926; Simmons *et al.*, 1931; Taniguchi *et al.*, 1951; Wisseman *et al.*, 1966).

IX. Conclusion

Dengue and dengue-like illnesses have been described in the medical literature for hundreds if not thousands of years. Dengue viruses have been introduced and re-introduced into regions of the world via exploration, the slave trade, war, the exportation and importation of goods, and tourism. Communities afflicted with dengue experience economic, social, and medical resource hardship. The successive circulation of different dengue virus serotypes throughout a region is believed to be responsible for the dramatic increase in severe disease (DHF) recently observed in Southeast Asia and the Americas. Although no cure exists, case-fatality rates have dropped significantly

in endemic countries due to careful clinical management. Numerous questions concerning the epidemiology and pathophysiology of dengue still remain. The development of a tetravalent dengue vaccine and its widespread use in dengue-endemic countries remains the most promising method to significantly reduce the global morbidity and mortality inflicted by this virus. Combined with effective vector control, the use of an effective vaccine would lead to a strong possibility of eradicating the virus from entire regions by a combination of increasing the herd immunity and decreasing the transmission potential.

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ADVANCES IN VIRUS RESEARCH, VOL 61

EPIDEMIOLOGY AND ECOLOGY OF YELLOW FEVER VIRUS

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I. History

Yellow fever (YF) virus has played an important role in the history of animal virology. There are reports of diseases with clinical descriptions similar to YF in 1498 in San Domingo and 1585 in West Africa (Scott, 1939). Subsequently, the first apparent epidemics of YF were recorded between 1647 and 1649 in Barbados, Cuba, and Mexico. The virus was first recognized as a human disease of major public health importance in the eighteenth century with large epidemics reported in both the

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Old World and New World. In addition, epidemics were also reported in Europe following importation of the virus in sailing ships. However, Griffin Hughes is thought to have been the first to use the term "yellow fever" in his book *Natural History of Barbados* (1750).

There were many debates over the agent and transmission of YF at the end of the nineteenth and start of the twentieth centuries. Carlos Findlay was the first to propose that mosquitoes transmitted YF. Subsequently, pioneering studies by Walter Reed and co-workers identified that the agent that caused YF was filtrable (i.e., it was a virus) and was transmitted by the bite of the mosquito *Aedes aegypti*. Thus, YF virus was the first animal virus shown to be transmitted by an arthropod. The virus itself was not isolated until 1927 when French and American researchers in Africa isolated the strains French viscerotropic and Asibi, respectively. Strain Asibi was isolated in Ghana, West Africa, from the blood of a male patient with fever, headache, backache, and prostration while the French viscerotropic virus was isolated from a mild case of YF in a Syrian man, Françoise Mayali, in Senegal. During the 1930s, both of these wild-type strains were used to derive live attenuated vaccines known as the French neurotropic vaccine and 17D, respectively (reviewed by Barrett, 1997 and Monath, 1999). The first South American strain, JSS, was identified in Brazil in 1935 and the jungle transmission cycle involving sylvatic mosquitoes and non-human primates was first identified in Brazil in 1932.

II. Classification

YF virus is the prototype virus of the family *Flaviviridae* that takes its name from the latin for yellow (*flavus*). The virus is a member of the genus *Flavivirus* that contains 67 human and animal viruses. Initially, the genus was divided, on the basis of plaque reduction neutralization tests, into eight serocomplexes and 17 viruses, including YF virus, that were antigenically distinct from other members of the genus (Calisher *et al.*, 1989). Recently, nucleotide sequencing of a region of the nonstructural protein NS5 gene of most flaviviruses has resulted in identification of genetic relationships that closely follow those of serologic relationships (Kuno *et al.*, 1998). However, the genetic analysis has allowed more detailed relationships to be established than was possible with serologic studies. The studies of Kuno *et al.* (1998), and subsequently by Gaunt *et al.* (2001) indicate that, on the basis of genetic homology, YF virus is closely related to nine other flaviviruses (Banzi, Bouboui, Edge Hill, Jugra, Saboya, Potiskum, Sepik, Uganda S, and Wesselsbron

viruses). Interestingly, Sepik virus (found in New Guinea) is genetically the most closely related to YF virus rather than African flaviviruses.

III. Ecology of Yellow Fever Virus

On the basis of their ecology, flaviviruses have been termed "*arbo*-viruses," or arthropod-borne viruses, to denote the fact many are transmitted between vertebrate hosts by mosquitoes or ticks. In the case of YF virus, the vertebrate hosts are primates while the arthropod hosts are normally mosquitoes. Mosquitoes are infected for life and thus can be considered to be the reservoir for the virus while monkeys have brief viremias and can be considered to be amplifying hosts.

A. Transmission Cycles

The virus is transmitted between primates by diurally active tree-hole breeding mosquitoes. Two transmission cycles are recognized. In the "Jungle" or "Sylvatic" cycle, the virus is transmitted among monkeys by tree-hole breeding mosquitoes. Humans are infected incidentally when entering the area (e.g., to work as foresters) and have what is termed "jungle yellow fever" (Fig. 1). The main vector in Africa is *Aedes africanus*, while in South America it is *Haemagogus* species. Other mosquito species involved in transmission include *A. africanus*, *A. furcifer*, *A. vittatus*, *A. luteocephalus*, *A. opok*, *A. metallicus*, and *A. simpsoni* in Africa, and *Sabethes chloropterus* in South America. The primate species acting as vertebrate hosts of the virus also differ by geographic area (see subsequent discussion). The "Urban" cycle involves transmission of YF virus between humans by *A. aegypti*, a domestic vector that breeds close to human habitation in water, and scrap containers including used tires, in urban areas or dry savannah areas. In this situation, the disease is known as "urban yellow fever" (Fig. 1). Although urban yellow fever is frequently reported in Africa, there have been no clearly documented cases of urban yellow fever in the Americas since 1942, or 1997 if a small cluster of cases in Santa Cruz, Bolivia (van der Stuyft *et al.*, 1999), is included, even though *A. aegypti* has reinfested all tropical South American countries in the past 20 years. In Africa, a third cycle is recognized, the intermediate or savannah cycle, where humans in the moist savannah regions come into contact with the jungle cycle. This has been referred to as the "Zone of Emergence." Although YF is considered to be a mosquito-borne disease, *Amblyomma variegatum* ticks have been shown to be

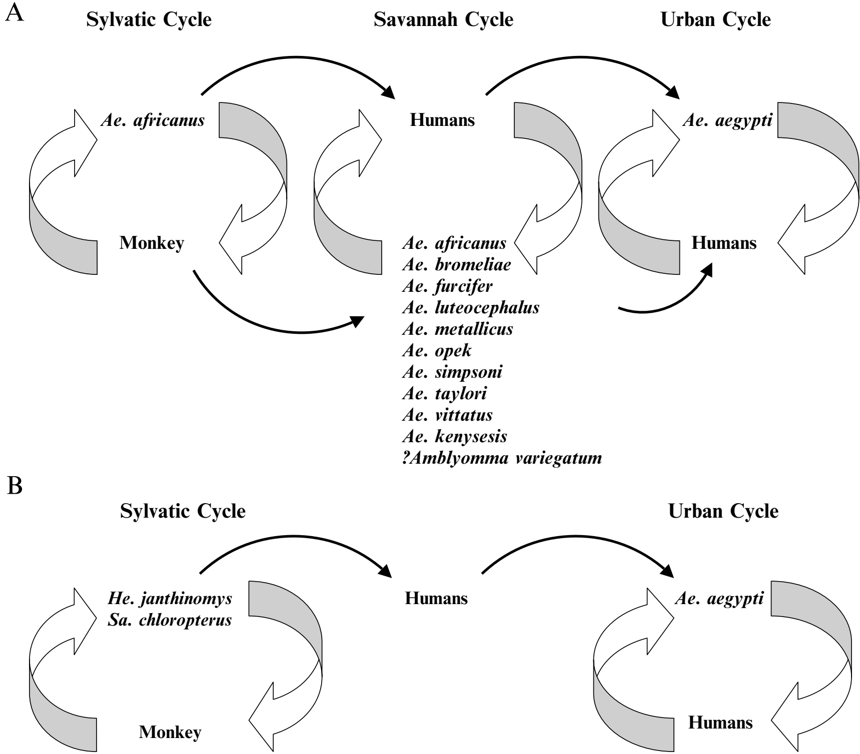

FIG 1. A. YF transmission cycles in Africa. B. YF transmission cycles in South America.

naturally infected with the virus in central Africa (Germain *et al.*, 1979). The significance of this observation in the ecology of YF virus has yet to be determined.

B. Arthropod Hosts

Arthropods (mosquitoes and ticks) are involved in biologic transmission of the virus (i.e., virus multiplies in the arthropod). Specifically, the arthropod acts as a vector to transmit the virus between vertebrate hosts. Vertical (transovarial) transmission enables the virus to survive in eggs during the dry season. If a female mosquito bites a virus-infected vertebrate that has a viremia greater than 10^4 pfu/mL, the virus is transmitted to the arthropod when it takes its blood meal. Virus infects the midgut epithelium and disseminates from the midgut

into the hemolymph and subsequently other tissues, in particular salivary glands and reproductive tract. When the mosquito takes its next feed, virus present in the salivary glands is injected into a new vertebrate host. It takes between 7 and 14 days for virus to be taken up during one feeding and spread to the salivary glands for transmission to a susceptible vertebrate host (i.e., the extrinsic incubation period). Thus, although the viremic phase in monkeys is short (approximately 3 days), monkeys act as an amplifying host for the virus and potentially many mosquitoes can be infected during the viremic phase. Transovarial transmission is considered to be an important component of the ecology of YF virus as infection of the mosquito reproductive tract enables eggs to become infected and allow "overwintering" of the virus when the climate is dry and/or cold. In particular, *Aedes* and *Haemagogus* eggs have been shown to survive desiccation and hatch with the return of the rainy season. Drought-resistant adult *Sabethes chloropterus* may also survive across the dry season, providing an alternative mechanism for virus persistence in nature, and ticks may play a similar role in Africa.

C. Vector Competence

Knowledge of vector competence of YF virus is limited. Many of the vectors associated with transmission of YF virus cannot be colonized (e.g., *H. janthinomys*), while there have been detailed studies on *A. aegypti* due to the ability to colonize this mosquito. Unfortunately, these studies have used artificial blood meals and a limited number of strains (mostly West African plus a few South American strains). These studies have demonstrated that viremias greater than 10^4 pfu/mL are required to infect mosquitoes and the ability of *A. aegypti* to transmit the virus to uninfected mice is variable (30–80%). Given the recent identification of multiple genotypes of virus (see subsequent discussion), there is a need to revisit vector competence of YF virus. Nonetheless, studies in Africa have shown that *A. aegypti* is an inefficient vector even in large epidemics such as those seen in Nigeria and Senegal. Despite this, the anthropophilic nature of the vector, and the very high densities of the mosquito in urban areas, enable it to be a vector for urban YF. It was hypothesized that the biological filter provided by a vector strain with low competence for virus transmission, may select for virulent virus strains capable of eliciting high viremias in humans (Miller *et al.*, 1989). Zoophilic *A. aegypti formosus*, which are prevalent in rural areas of Africa, do not appear to be involved in transmitting YF virus in the sylvatic cycle.

YF virus has been isolated from *Amblyomma variegatum* in the Central African Republic (Germain *et al.*, 1979). Vertical transmission of virus indicates that ticks may play a role in the maintenance of the virus in nature.

D. Vertebrate Hosts

Although primates are the vertebrate host of YF virus, the virus infects a number of animal species. The virus causes a viscerotropic (i.e., infection of liver, spleen, heart and kidneys) disease in humans, monkeys and European (*Atelerix erinaceus*) and Sudanese (*Atelerix pruneri*) hedgehogs. Recent work has shown that some strains of YF virus will cause hepatotropic disease in Syrian hamsters (Tesh *et al*. 2001). The primary tissue tropism of the virus is the liver for the above animals, and is not associated with encephalitis. However, the virus causes neurotropic disease in mice, hamsters, and guinea pigs, usually only following intracerebral inoculation. A number of vertebrates (e.g., sloths, and neotropical marsupials) have been found to have antibodies against YF virus, and the virus has been isolated on a single occasion from a fruit bat in East Africa. Considerable attention was given to neotropical marsupials in early studies on experimental infection of wild-caught species. These studies showed that the animals seroconverted and had low viremias but these were considered generally insufficient for mosquitoes to become infected on feeding. Nevertheless, certain species, such as *Metachirus nudicaudatus*, developed viremias sufficient to infect *Haemogogus* (Bates and Roca-Garcia, 1946)

Only primates appear to be clearly implicated vertebrate hosts in the transmission cycles of YF virus. In general viremias are short in duration (2–5 days), although it can be 9 days in *Colobus abyssinicus*. Non-human primates either die following infection with the virus or develop life-long protective immunity.

Although African monkeys are susceptible to YF virus and become viremic, in general they do not show clinical signs of infection. *Colobus abyssinicus* is the major non-human primate species for YF virus in East and Central Africa while *Cercopithecus* spp. are vertebrate hosts in various forest and savannah areas in Africa. *Galago senegalensis*, a member of the suborder *Lemurioidea*, is found in many parts of West, Central, and East Africa. Interestingly, serological studies suggest that *G. senegalensis* is an important vertebrate host in East Africa and, unlike other non-human primates in Africa, develops clinical

disease, including liver necrosis and fatal disease; however, this species does not appear to be involved in the transmission cycle in West Africa. The latter may be due to mosquito vectors in West Africa not feeding on *G. senegalensis* or genetic differences between virus genotypes found in West and East Africa.

E. Role of Climatologic Factors

The ecology of YF virus is complex with many factors contributing to the possibility of YF activity. Yellow fever activity often occurs in areas after increases in temperature and rainfall that will favor increased biodiversity, including increased numbers of animals and arthropods while reduced rainfall limits mosquito vector density. It has been known for over 50 years that increased temperatures are associated with enhanced transmission of YF virus (Strode, 1951) due to shortened extrinsic incubation period and increased biting by mosquitoes of vertebrate hosts. One of the most fruitful studies on YF in West Africa has been undertaken by French and Senegalese workers in the Kedougou region of eastern Senegal and have shown that outbreaks of YF over wide areas of West Africa correlate with high isolation rates of YF virus from mosquitoes in the localized region in eastern Senegal. This correlation is consistent with the hypothesis that an increase in the force of infection in the sylvatic cycle leads to a higher probability of virus infection of humans and that ecological factors affecting large areas are responsible. The reason(s) for the increase in the sylvatic cycle have been subject of conjecture in recent years and increased rainfall, temperature, El Nino, and other factors have all been proposed. Similarly, Monath (1995, 1997) showed that increased rainfall and prolonged rainy season, seen as increased vegetation densities as determined by Geographic Information Systems, preceded the YF epidemic in Nigeria in 1986 and that El Nino events were temporally associated with periodic YF emergences in West Africa. In addition, global warming increases areas where vectors are found such that the potential geographic area of YF virus activity is increasing. In contrast to increased rainfall, drought conditions can also contribute to YF activity as reduced availability of water sources encourages animals to share water sources so that introduction of a viremic host to an area with many susceptible hosts can rapidly result in transmission of virus between vertebrate hosts. This is thought to have been a contributing factor to the outbreak in Kenya in 1992–1993, which occurred after a 56-year absence of YF disease in that country.

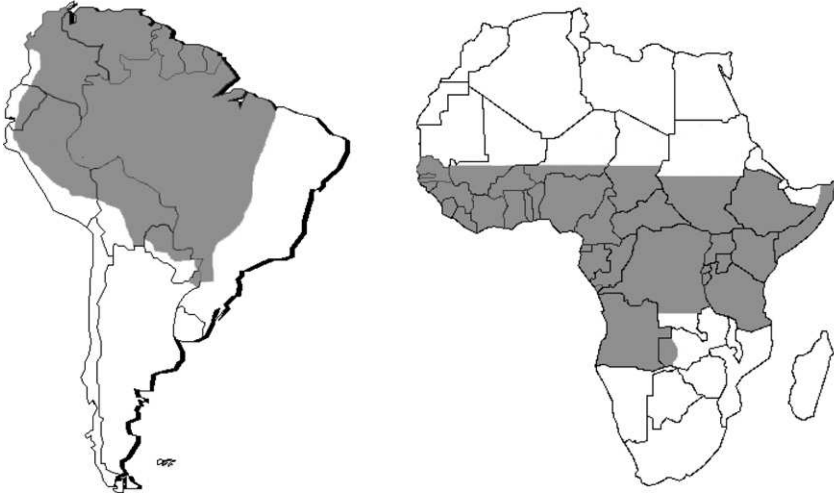

FIG 2. Geographic distribution of yellow fever virus in Africa and South America.

IV. EPIDEMIOLOGY OF YELLOW FEVER VIRUS

A. Geographic Locations of Yellow

YF virus is only found in tropical South America and sub-Saharan Africa (Fig. 2), including 34 countries in Africa and 14 countries in South America; the virus is not found in Asia. YF virus is considered to be an epizootic virus due to the fact that the virus normally infects monkeys in nature. However, the virus has been shown to be endemic and epidemic depending on the geographic location. Current YF activity is reported in the World Health Organization *Weekly Epidemiological Record*. It should be emphasized that this is the "official" record and probably greatly underestimates the number of YF cases as these official reports usually only identify hospitalized cases and do not take into account the large numbers of mild cases. Furthermore, most reports of YF refer to epidemic disease and do not take into account endemic disease. Most YF outbreaks take place in remote areas where the disease is only recognized late into the outbreak and diagnosis is initially made on the basis of clinical disease with laboratory confirmation of disease being made in many instances after the outbreak. In this situation, it is very difficult to measure the extent of endemic disease, particularly if patients have mild forms of the disease. Overall, it is estimated that there are approximately 200,000 clinical

cases each year of which the vast majority (>90%) of cases occur in West Africa (Robertson *et al.*, 1996). The case-fatality rate is approximately 20%. There have been few attempts to estimate the infection:illness ratio, but Monath and Nasidi (1993) estimated that in Nigeria one in seven of those infected will become ill, while 0.1–0.2% of those infected will get jaundice and 0.02–0.05% will have a fatal infection.

B. Africa

YF is endemic in many parts of West, Central, and East Africa, principally between latitudes 15°N and 15°S. The majority of outbreaks have been reported from West Africa whereas there have been much fewer outbreaks in central and east Africa. This may be due to a combination of factors, including large population densities for both humans and mosquito vectors, low vaccine coverage and high potential for inter-human transmission by *A. aegypti*. The areas where the virus is found correspond to rainforest or moist savannahs. Peak activity in the moist savannas takes place in the mid-rainy to early dry season (August–October), where *A. furcifer* and other species are seasonally abundant; transmission occurs throughout the year in the rainforest, where *A. africanus* is the principal vector.

1. West Africa

Epidemics of YF, including urban YF, have been known in West Africa for many centuries. Epidemics in the twentieth century have contributed greatly to our knowledge of the epidemiology of YF. The 1927–1928 epidemic was significant as it was the first isolations of YF virus, namely Asibi and the French viscerotropic virus. These isolations subsequently led to the development of live attenuated vaccines, 17D and French neurotropic vaccine (FNV), respectively, that in concert with anti-mosquito campaigns resulted in a huge reduction of YF cases to the extent that sporadic cases were only reported. However, vaccination programs were curtailed in the early 1960s as the disease was controlled and there was increased focus on vaccine safety issues. During mass vaccination campaigns particularly in response to an epidemic of YF in Senegal in 1965, there was a high incidence of serious adverse events (post-vaccinal encephalitis) associated with the use of FNV in children. The reactogenicity of FNV had been recognized before, but largely ignored due to the urgency to protect the population against YF. As the use of FNV declined after this episode, a large childhood population of immunologically susceptible children arose in francophone West Africa, leading to re-emergence of epidemic YF in

the mid-1980s. In anglophone West Africa, FNV was never accepted for use (because of safety concerns), and YF 17D vaccine was generally not available, so that the population has sustained repeated outbreaks of YF over the years. Nigeria and Ghana have sustained repeated well-documented epidemics of jungle, savanna, and urban YF during the last 50 years of the twentieth century, with an especially intensive series of outbreaks and an increase in reported cases between 1986 and 1991 (Nasidi *et al.*, 1989; Robertson *et al.*, 1996). Serological surveys have shown that monkeys and humans in many parts of Nigeria have antibodies to YF virus indicating widespread transmission of the virus in Nigeria. Not surprisingly, there are continual reports of YF activity in Nigeria. In comparison, YF virus exists in endemic foci in many countries in West Africa, with low rates of seroconversion in humans and sporadic human cases, and occasional epidemics.

Although virus has also been demonstrated in at least 10 mosquito species, all in the *Aedes* genus, *A. africanus*, a canopy-dwelling mosquito is considered the major vector of YF virus in West Africa. This may be due in part to its presence not only in jungle but also on the edge of forests. *A. furcifer*, *A. taylori*, *A. opok*, *A. metallicus*, *A. vittatus*, and *A. luteocephalus* are vectors in savanna areas, and together with the urban vector *A. aegypti*, are considered to be other significant vectors in Africa.

Urban outbreaks still occur in West Africa (most notably in Nigeria). Outbreaks usually start as jungle YF outbreaks due to movement of humans into forested areas in search of food or employment (e.g., deforestation). Infected individuals carry the virus to villages outside jungle areas and transmission takes place and it is easy to envisage how an outbreak could begin as a jungle outbreak and rapidly become an urban outbreak.

2. Central Africa

In central Africa, sporadic cases are reported from Democratic Republic of Congo (formally Zaire), Cameroon, Central African Republic, and Angola. In the Central African Republic, epizootics take place every 4–5 years involving *A. africanus* and *A. opok*.

3. East Africa

Outbreaks in East Africa are few and far between (Table I). For example, YF outbreaks were reported in Sudan in 1940 and 1959, in Ethiopia in 1960 and 1966, and in Kenya in 1936 and 1992–1993, but no human disease was reported in the other years. Studies subsequent to the 1993 outbreak discovered that people were seropositive to YF virus indicating that inapparent or mild infections were taking place.

TABLE I
OUTBREAKS OF HUMAN YF IN EAST AFRICA

Year	Countries
1912	Zaire
1917	Zaire
1927–1928	Zaire
1936	Sudan, Uganda, Kenya
1940	Sudan
1958	Zaire
1959	Sudan
1960–1962	Ethiopia
1966	Ethiopia, Sudan
1971	Angola
1972	Zaire
1988	Angola
1992–1993	Kenya

A similar situation has been reported in Uganda where sylvatic transmission among monkeys could be demonstrated by seroconversion while humans remained seronegative. There are three potential explanations for these findings. First, the virus strains found in East Africa could be less virulent than those found in West Africa and South America. Genotypic differences in viruses found in these three geographic regions would support this hypothesis (see later). Second, the mosquitoes transmitting the virus may be inefficient at transmitting the virus or do not feed on humans. This hypothesis could be supported by demonstration that *A. kenysesis* and *A. simpsoni* complex (e.g., *A. bromeliae*) mosquitoes are vectors in East Africa whereas *A. africanus* is found throughout areas of sub-Sarahan Africa where the virus is found. Third is the presence of antibodies against other flavivirus infections that may contribute to cross-protective immunity.

C. South America

The majority of YF activity in South America is found in the Orinoco, Amazon, and Araguaia river basins with occasional reports from surrounding areas, including Trinidad. Approximately 200 cases of jungle YF are reported from South America each year with a case-fatality rate of 65%. This high figure is in part due to identification of the disease in some areas by histopathologic examination of livers from fatal cases. This has led to speculation that the true incidence of YF

may be 10-fold greater than reported numbers of cases. However, it should be remembered that YF takes place in jungle areas where the human population density is very low. Most YF cases are reported in Peru and Bolivia and involve males aged 15–45 years who are agricultural and forest workers. YF usually occurs from December through May and peaks during the first 3 months of the year when populations of *Haemagogus* mosquitoes are highest during the rainy season.

Historically, YF has been reported from many counties in tropical South America from Panama in the north to Argentina in the south. However, most activity is reported from Bolivia, Brazil, Colombia, Ecuador, the Guianas, Peru, and Venezuela. In most situations, human cases have been sporadic and involve individuals who enter forest areas containing the mosquito vector *H. janthinomys*. Vaccination is used to control outbreaks, and in recent years this has been very effective at reducing the number of human cases.

However, Peru, Brazil, and Bolivia account for approximately 90% of cases reported in South America. In Brazil the vast majority of cases are reported from the Amazonian and central western areas of Brazil. However, recently cases have been reported outside this region in São Paulo, Bahia, Tocantins, and Goias states. Also, in 1998, the first cases of YF were reported in French Guiana since 1902 (Heraud *et al*., 1999). Thus, YF is a reemerging disease in tropical South America.

In 1949, 10 countries in tropical Central and South America (Bolivia, Brazil, Guyana, Colombia, Ecuador, French Guiana, Panama, Peru, Surinam, and Venezuela) established a program to eliminate *A. aegypti*. This program was very successful and resulted in the elimination of the mosquito from urban areas, and with it urban YF (Maurice, 1993). However, the number of YF cases reported from South American countries increased from 1985, including in 1995 an outbreak in Peru involving at least 790 cases with case-fatality rate of 38%, which was the largest outbreak in South America since the 1950s. In addition to the increased numbers of cases in recent years, *A. aegypti* has reinfested many urban centers in South America and there is now the potential that urban YF will return to the Americas.

Studies during the 1940s and 1950s in Brazil, Trinidad, and Colombia by the Rockefeller Foundation established the role of non-human primates and forest canopy breeding mosquitoes in the jungle cycle, and the apparent cyclic nature of YF epizootics where virus appeared to cause outbreaks followed by 4–7 years where no virus activity was reported. These studies gave rise to the prevailing paradigm that YF virus is maintained by wandering epizootics through the Amazon basin in a sylvan cycle involving non-human primates and mosquitoes

of the *Hemagogus* and *Sabethes* genera rather than the virus remaining in one locale. *H. janthinomys* is highly susceptible to YF virus and is primatophilic. Thus, it is not surprising that humans entering the *H. janthinomys* ecosystem have a high chance of being bitten and infected with YF virus. Nearly all monkey species are susceptible to YF virus with the potential of a fatal outcome and traditionally the finding of dead monkeys has been the marker for a YF epizootic. However, given that monkeys either die from YF virus infection or become immune to infection, and the long gestational period and low population turnover of monkeys, and the absence of alternative vertebrate hosts, the paradigm of virus movement to sustain the jungle cycle has become the established hypothesis to explain YF activity in tropical South America. Subsequent studies have established a cyclic pattern of YF activity with inter-epidemic intervals considered as the time required to establish susceptible populations of non-human primates. Recent studies by Vasconcelos *et al*. (1997, 2001a, 2001b) of YF outbreaks in Brazil have suggested focal endemism of YF virus in areas of Brazil on the basis of annual isolation of virus from *H. janthinomys* during the rainy season near the TransAmazon Highway. Similarly, the pattern of sylvan YF activity observed in the Amazon region of Peru and Bolivia suggests that the "epizootic waves" model may not be valid for eastern Peru and Bolivia. Peru and Bolivia have accounted for 85% of the total cases of YF reported from South America during the past decade. Because YF surveillance activities are coordinated in South America by the Pan American Health Organization, it is unlikely that the large number of cases reported from Peru and Bolivia are the result of discrepancies in reporting cases. Thus, it is assumed that the differences in numbers of cases are real. There are areas in eastern Peru and Bolivia where human cases of YF occur every year, suggesting that enzootic foci may exist in these countries.

D. Why Has Yellow Fever Never Been Seen in Asia?

The lack of yellow fever virus in Asia is not understood, although a number of hypotheses have been put forward (Monath, 1989). The mosquito vector *A. aegypti* is prevalent in Asia, and laboratory studies indicate that Asian strains of *A. aegypti* can transmit YF virus, albeit less competently than strains from the Americas. Demographic factors, principally the remote location of sylvatic YF transmission, and cross-protective immunity provided by previous exposure to dengue are considered likely to play a role. This topic is discussed in more detail in the chapter entitled "Origins, Evolution, and Vector/

Host Co-adaptations Within the Genus *Flavivirus*" by Ernest A. Gould *et al.*, in Volume 59 of this series.

V. Molecular Biology of Yellow Fever Virus

A. Antigenic Variation

Clarke (1960) was the first to identify antigenic differences between strains of YF virus based on antibody absorption when she demonstrated differences between African and South American strains and described three "groups": West African, East African, and South American. The advent of monoclonal antibodies (MAbs) enabled more precise differences to be elucidated. MAb reactivity with epitopes on the nonstructural protein NS1 and envelope (E) proteins demonstrated that vaccine and wild-type strains could be distinguished and wild-type strains from Africa and South America could be distinguished (Buckley and Gould, 1985; Deubel *et al.*, 1987; Gould *et al.*, 1985, 1989; Schlesinger *et al.*, 1983; Sil *et al.*, 1990). Polyacylamide gel electrophoresis showed that mobility of the E protein varied between strains and this was subsequently shown to be due in part to differences in glycosylation (Cane and Gould, 1989; Deubel *et al.*, 1987). South American strains had two glycosylation sites on the E protein while African strains had one or none. Biological studies showed that virulence of wild-type strains for mice varied. In particular, lethality following intraperitoneal inoculation of 8-day-old mice proved to be a significant marker as South American strains were virulent while some African strains were not (Deubel *et al.*, 1987; Fitzgeorge and Bradish, 1980). However, wild-type YF virus is only neurotropic in mice and this is not the normal tropism of wild-type YF virus; thus although this is a phenotypic difference, the biologic significance of these results is unclear.

B. Genome of Yellow Fever Virus

The genome consists of a single-stranded, positive-sense RNA of approximately 11,000 nucleotides. The genome of the prototype strain Asibi is 10,862 nucleotides in length (Hahn *et al.*, 1987; Rice *et al.*, 1985). The 5′ terminus of the genome posesses a type I cap (m^7 GpppAmp) followed by the conserved dinucleotide AG. There is no terminal poly (A) tract at the 3′ terminus. The gene order is C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-2K-NS4B-NS5 (Rice *et al.*, 1985). The 5′

noncoding region is nearly identical in sequence for all strains studied, while the 3′ noncoding region includes imperfect repeat sequences of 42 nucleotides. South American strains have one copy of the repeat, while East African strains have two copies and West African strains have three copies. The repeat sequences are thought to be due to the evolution of YF virus in Africa followed by the introduction of YF virus into South America from Africa. Because South American strains of YF virus only have one copy of the repeat sequence, only one copy must be required for replication of the virus.

The genetic database for YF virus is very limited. The nucleotide sequence of the genome of the Asibi strain was determined in 1987 (Hahn *et al.*, 1987). Since then, the complete genomes of only two other African YF virus strains have been determined; namely, the French viscerotropic strains isolated in Senegal in 1927 (Wang *et al.*, 1995) and strain 85-82H isolated in the Ivory Coast in 1982 (Pisano *et al.*, 1997) plus the Trinidadian strain 788379 (Pisano *et al.*, 1999). Strain 788379 had extensive nucleotide homology with West African strains and the authors speculated that this strain was an example of recent introduction of YF virus into Trinidad from West Africa. There are no genomic sequences representing strains from East and Central Africa. Ballinger-Crabtree and Miller (1990) reported the first nucleotide sequence data for a South American strain when they showed that strain 1899/81 from Peru differed by 14.7% in the structural protein genes from West African strain Asibi, including an additional glycosylation site in the E protein.

C. Molecular Epidemiology

The first genetic studies were undertaken by Deubel *et al.* (1985, 1986) who used T1 RNA oligonucleotide fingerprinting (a technique that evaluates approximately 10% of the genome) and found that at least four topotypes (or geographic variants) existed: Senegal-Gambia and Côte D'Ivoire-Burkino Faso-Nigeria in West Africa, Central and East Africa, and South America. The viruses belonging to the Senegal topotype were very homogeneous even though they were isolated over a 50-year period (Deubel *et al.*, 1985). Examination of three South American strains suggested a single topotype in South America.

Deubel's group (Lepineic *et al.*, 1994) was the first to apply nucleotide sequencing to study the molecular epidemiology of YF virus. Examination of a portion of the envelope (E) protein gene revealed the identification of three genotypes in Africa; two in West Africa and one in East Africa, while inclusion of one South American strain (1899/

81 from Peru) sequenced by Ballinger-Crabtree and Miller (1990) indicated at least one genotype in South America. The following year, Chang *et al*. (1995) compared the nucleotide sequence of the entire E gene of a number of YF virus strains. Their conclusion was somewhat different from that of Lepineic *et al*. (1994) and proposed three genotypes: one in West Africa, one in East Africa, and another in South America. A subsequent study by Wang *et al*. (1996) proposed four genotypes; one each in West and East Africa, and two in South America.

Six studies (Chang *et al*., 1995; Duebel *et al*., 1985, 1986; Lepiniec *et al*., 1994; Wang *et al*., 1996, 1997) have specifically analyzed genetic and phylogenetic relationships among relatively few YF strains: three (Wang *et al*., 1997) to 21 (Lepineic *et al*., 1994). This is underrepresentative, considering the geographic spread of the virus and frequency of YF outbreaks. Most of these studies (Chang *et al*., 1995; Duebel *et al*., 1986; Lepiniec *et al*., 1994; Wang *et al*., 1996) showed clear genetic and phylogenetic distinction between East/Central and West African YF strains. However, the samples were biased toward West Africa, which is probably attributed to the availability of isolates due to more YF activity in this region compared to East and Central Africa. For example, Lepiniec *et al*. (1994) analyzed 21 wild strains of YF virus, but only six (28.5%) were from East and Central Africa. Nonetheless, there was clear evidence of a low rate of evolution of strains from the same geographic area. For example, comparison of strains isolated in either Senegal or the Central African Republic over a 14-year period had less than 1% nucleotide variation (Lepineic *et al*., 1994). Also, two Nigerian strains isolated in 1986 and 1991 differed by only 0.3% in their E gene sequence (Chang *et al*., 1995). Not surprisingly, strains isolated from the same outbreak or epidemic are very closely related at the genetic level (Chang *et al*., 1995; Lepineic *et al*., 1994; Wang *et al*., 1996).

Mutebi *et al*. (2001) examined a large panel of 37 wild-type YF virus strains from Africa to elucidate the precise genetic relatedness of wild strains of YF virus in Africa. These studies identified five genotypes in Africa, two in West Africa (as proposed by Lepineic *et al*., 1994), and three in Central and East Africa (termed East Africa, East and Central Africa, and Angola) (Fig. 3). West Africa genotype II (similar to the Senegal topotype of Deubel *et al*. [1985]) was very homogeneous and strains showed only 2.8% nucleotide variation even though they were isolated over 65 years, whereas West Africa genotype I (similar to the Nigeria topotype of Deubel *et al*. [1986]) revealed up to 6.8% nucleotide variation over 45 years. The situation in East and Central Africa was surprising. One genotype was found in East

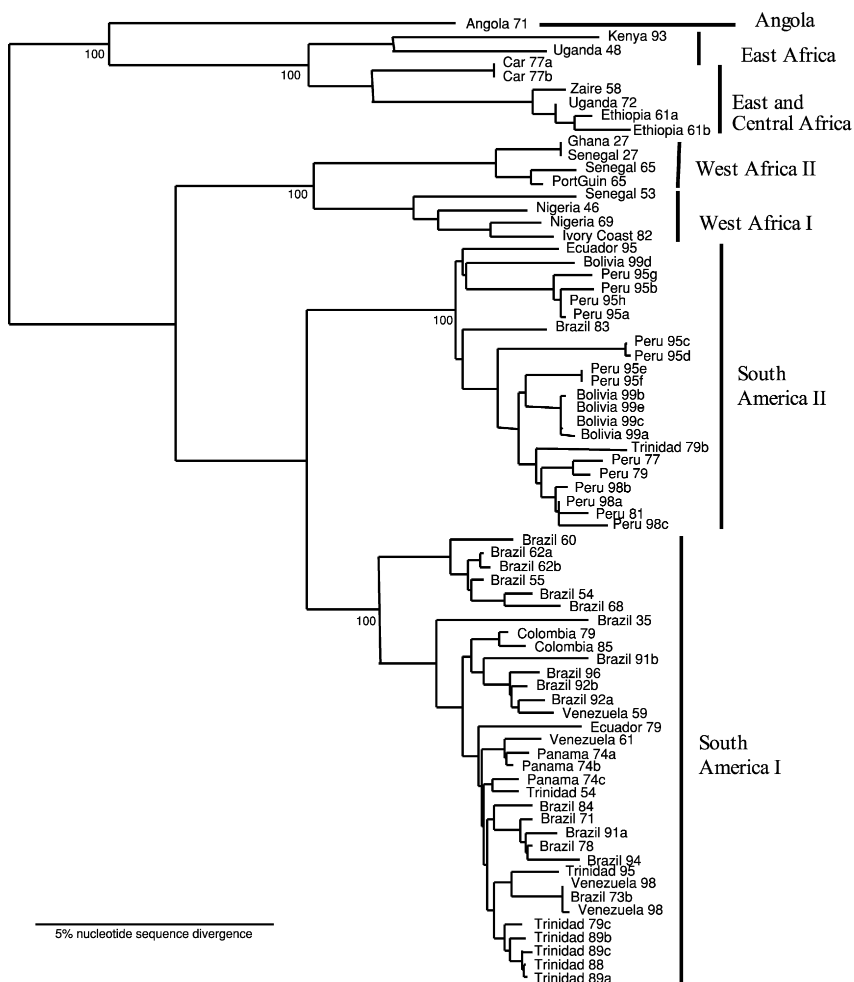

FIG 3. Phylogenetic tree derived of 73 yellow fever virus strains from Africa and South America. The tree was constructed using the neighbor joining program. Strains are designated by country and year of isolation. Bootstrap values are shown for groups to the right.

and Central Africa that encompassed a large geographic area including Sudan, Ethiopia, Central African Republic, Democratic Republic of Congo (formally Zaire), and Uganda over a 50-year period. As stated previously (Section IV,B,3), epidemics in these counties are infrequent and separated by long periods without human outbreaks.

Epidemiologic studies had suggested that epidemics in these countries were due to introduction of epidemic strains from western Africa while the molecular characterization indicates that the epidemics are due to virus strains that are probably continually present in sylvatic cycles in these countries. The Angola genotype was also surprising. The single strain of virus from Angola was isolated in 1971 during an urban outbreak of YF that was the first reported outbreak in Angola for 99 years. Previously, the 1971 outbreak had been considered due to importation of an epidemic strain of YF virus from West Africa while the genetic studies indicate that this outbreak was due to a sylvatic strain. Furthermore, the Angolan strain appears to be a member of a distinct lineage that has been evolving separately from the other East and Central African viruses for a long time based on its basal position in phylogenetic tree of African viruses. It is conceivable that more genotypes of YF virus will be discovered in Africa from sylvatic cycles and this may help in understanding the evolution of YF virus.

Previous nucleotide sequencing studies have only examined a few isolates of YF virus from South America. In one study, Lepeniec *et al.* incorporated a single Peruvian strain (1899/81) that had been previously described by Ballinger-Crabtree and Miller (1990). In a second study, Chang *et al.* (1995) included eight South American strains among a total of 20 YF viruses examined. On the basis of the latter work, Chang *et al.* (1995) identified one South American genotype. In contrast, Wang *et al.* (1996) examined a panel of 14 YF virus strains, including seven from South America. Based on 1320 nucleotides from the structural protein genes, four genotypes were proposed. This included one from West Africa and another from East/Central Africa, as proposed previously (Chang *et al.*, 1995; Lepineic *et al.*, 1994). However, Wang *et al.* differed from the above two groups in proposing that two genotypes of YF virus existed in South America. Genotype I included viruses from Trinidad, Brazil, and Panama, while genotype II included two isolates from Peru and one from Trinidad. The origins of the two South American genotypes were unclear as the oldest South American genotype II virus was isolated in 1977 while the oldest South American genotype I virus was from Brazil in 1935 and was the first isolate made in South America. Thus, more studies are necessary to delineate the relationships of the two South American genotypes. Recent studies by Bryant, Wang, and Barrett (unpublished; Fig. 3) have examined a larger panel of South American strains and confirmed that two genotypes exist in South America as proposed by Wang *et al.* (1996).

Unlike previous studies, Wang *et al.* (1996) examined different portions of the genome of the 14 strains studied: 1320 nucleotides from

the structural protein genes (i.e., the 5′ end of the genome), 754 nucleotides from NS4A/NS4B genes (i.e., the middle of the genome), and the 3′ non-coding region (3′NCR). Phylogenetic analyses revealed that the trees generated for the 5′ region, middle of the genome and 3′NCR were very similar. On the basis of studying approximately 25% of the genome, it was concluded that the virus genome was evolving at a constant rate and that potentially any region of the genome could be used to examine genetic variation. Furthermore, it suggested that recombination was, at the very least, a rare event.

D. Origins of Yellow Fever Virus

The origins of YF virus have been the subject of great debate for many years. Based on clinical descriptions of disease the first report of YF may be as early as 1498 in San Domingo in Central America and 1585 in West Africa (Scott, 1939). These writings are suggestive that YF virus was found in the Americas before Africa, while the classical view has been that YF virus (and *A. aegypti*) originated in Africa and moved with the slave trade from West Africa to South America. Phylogenetic data support the view that YF virus originated in Africa and split into two lineages that were found in West Africa and East Africa. Subsequently, South American strains of YF virus were derived from a progenitor virus from West Africa (Fig. 3). This is also consistent with the so-called "Out of Africa" origin of the *Flavivirus* genus as a whole (Gould *et al.*, 2001). However, it is puzzling that Sepik virus (from New Guinea) is genetically the closest relative to YF virus (Kuno *et al.*, 1998). Clearly, further studies are warranted on the origin and evolution of YF virus.

VI. Reemergence of Yellow Fever as a Major Public Health Problem

YF remains a major public health concern in sub-Saharan Africa and tropical South America despite the availability of a safe and effective vaccine. For example, in the period between 1987 and 1991 a total of 18,735 YF cases and 4522 deaths were reported to the World Health Organization (Robertson *et al.*, 1996). These figures represent the greatest amount of YF activity since 1948 (Robertson *et al.*, 1996). More recent statistics reviewed by Vainio and Cutts (1998) indicate that the trend in increasing numbers of cases continues. Thus, YF is considered to be a reemerging disease due to increase in the incidence of the disease in the past 20 years.

To understand the cause of the reemergence, one must look to the middle of the 20th century. Up until the development of the live attenuated 17D and FNV vaccines, YF was considered a global public health problem and the disease was subjected to a very large research effort. The introduction of vaccination to control the disease had far-reaching consequences. On the positive side, one dose of vaccine was found to give apparent lifelong immunity and presented the opportunity to eliminate the disease from humans. YF 17D vaccine can be given to children aged nine months or older, and the resulting immunity is lifelong (Poland *et al.*, 1981). YF 17D vaccine has long enjoyed a reputation of being one of the safest and most effective live attenuated vaccines (Barrett, 1997; Monath, 1999). As such, the incidence of YF drastically reduced following the introduction of vaccination in the late 1930s and the next 25 years saw relatively few cases of YF with outbreaks limited to those countries that did not administer one of the vaccines. As the number of reported cases decreased, so did efforts to control the disease. Complacency set in, along with a feeling that YF was beaten. However, YF is an epizootic disease and vaccination could not eliminate the nonhuman primate host of the virus, and the potential was always present that the disease incidence would increase in humans if vaccination was not maintained to immunize susceptibles born after vaccination programs. Significantly, few countries, with the notable exception of Gambia in Africa, introduced the YF vaccine into their "Expanded Program of Immunization" (EPI) and the YF vaccine was not included in childhood vaccination programs. Thus, as vaccination campaigns were not maintained, numbers of cases of YF have increased, particularly in children and young males who enter jungle areas for employment (Tomori *et al.*, 1994; UNICEF, 1988). The late 1950s and early 1960s saw resurgence in the number of reported cases and several outbreaks were reported in Africa. The number of cases continues to increase as the EPI and routine vaccination campaigns have been replaced by emergency vaccination campaigns once an outbreak has been identified. Once the outbreak ceases, so does vaccination. Clearly, this is not a cost-effective mechanism to control a vaccine-preventable disease.

A. Risk of Urban Yellow Fever Returning to the Americas

Although *A. aegypti* was eliminated from many South American countries in the 1930s to 1940s, control measures were not maintained, and *A. aegypti* soon returned to the countries from where it was eliminated and today the mosquito infests a larger area of the Americas than before its temporary elimination. There are many

reasons for this, including political decisions not to maintain mosquito control programs and global warming where increase in temperatures and rainfall favor increases in populations of mosquitoes and other animals. However, the important point is that urban YF has not returned to the Americas while infestation with *A. aegypti* has been associated with ever-increasing rates of transmission of dengue viruses. There is one report of urban YF in the Bolivian city of Santa Cruz in 1997–1998 consisting of six serologically confirmed cases, including five deaths, that were restricted to a small neighborhood (Van der Stuyft *et al.*, 1999). Interestingly, no further urban YF has been reported in Santa Cruz, indicating that this small outbreak did not lead to larger outbreaks/epidemics. This may be due to increased vaccination coverage, as Bolivia has increased vaccine coverage in the last several years.

Many hypotheses have been proposed to explain the lack of urban YF and these are very similar to those proposed to explain the lack of YF virus in Asia (see Section IV,D). However, the general consensus is that it is only a matter of time before urban YF returns due to increasing sizes of urban human populations and geographic spread of *A. aegypti*. It is worth comparing YF and the four dengue viruses. All are mosquito-borne flaviviruses; however, the evolution of these viruses has been very different. All these viruses originated as sylvatic viruses putatively involving monkey-mosquito transmission cycles. YF virus has retained its sylvatic phenotype, whereas the dengue viruses have evolved into a human-mosquito (predominantly *A. aegypti*) transmission cycle. Furthermore, molecular epidemiologic studies show that YF virus has undergone relatively little genetic variation and correspondingly very few amino acid substitutions (see Section V,C). In comparison, the dengue viruses have undergone extensive nucleotide and amino acid variation (Rico-Hesse, 1990; Wang *et al.*, 2000). Thus, it is possible that the lack of urban YF may be in part due to YF virus retaining its sylvatic character and not adapting to *A. aegypti* while dengue viruses have evolved and followed *A. aegypti* in to South American urban population centers.

B. Ecotourism

One potentially important factor in the emergence of YF is ecotourism. People residing in developed countries are taking vacations to developing countries in increasing numbers, and residents of urban, coastal South America are vacationing in the endemic interior regions of the country. Unfortunately, many tourists are not vaccinated prior

TABLE II
RECENT EXAMPLES OF ECOTOURISTS ACQUIRING FATAL YELLOW FEVER INFECTIONS WHILE ON VACATION

Year	Home of Tourist	Country Visited by Tourist
1996	USA (Tennessee)	Brazil
1996	Switzerland	Brazil
2000	Germany (Berlin)	Cote d'Azore
2000	USA (California)	Venezuela
2001	Belgium	Gambia
2002	USA (Texas)	Brazil

to taking their vacation. There have been a number of incidences where tourists have been abroad and returned to their homes to succumb to YF (Table II). This has potentially important issues for public health. First, air travel enables viremic travelers to reach any part of the world in less than 24 hours while they are still viremic. Second, few physicians in developed countries have seen a case of YF and are not trained in caring for patients. Third, and most importantly, if the developed country has a mosquito vector that will transmit YF virus (e.g., *A. aegypti*) there is potential to establish large outbreaks of human disease and/or YF virus in the ecosystem. This is more than a theoretical risk as demonstrated by the introduction of another flavivirus, West Nile virus, into North America in 1999 (Lanciotti *et al*., 1999).

C. Future Control of Yellow Fever

YF is a vaccine-preventable disease and as such is a disease that should be controlled by an effective vaccination program. The ability of vaccination to control YF has been demonstrated in the 1940s to 1950s but a vaccination program must be sustained. The most cost-effective route to controlling the disease would be inclusion of the vaccine in the Expanded Program of Immunization and inclusion of the vaccine in childhood immunizations campaigns in tropical countries in Africa and South America where the virus is found. This goal has now been accepted by all countries in South America and is being steadily implemented, and some progress is also being made in Africa, with donations of funds for YF vaccine purchase from the Gates Foundation. However, populations of coastal areas of South America infested by *A. agypti* are not yet protected, and vaccination coverage rates in many African countries remain low. With worldwide air travel, ecotourism is a potentially very dangerous route to importation of YF

into large urban centers where *A. aegypti* is found. Physicians and immigration officials must encourage administration of YF vaccine to travelers from developed countries to developing countries in tropical regions of the World.

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ADVANCES IN VIRUS RESEARCH, VOL 61

TICK-BORNE FLAVIVIRUSES

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Tick-borne encephalitis (TBE), one of the most dangerous neuroinfections in Europe and Asia, is caused by tick-borne encephalitis virus (TBEV) and currently involves approximately 11,000 human cases annually, mostly in Russia. This chapter describes the main problems associated with the epidemiology, ecology, pathogenesis, and control of this

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disease. We have attempted to review the factors that influence the incidence and distribution of TBE, and to discuss possible reasons for the different clinical manifestations including most commonly observed asymptomatic infections, fever forms, acute encephalitis, and the less frequently registered biphasic milk fever and chronic encephalitis.

Epidemiologic data concerning the other tick-borne flaviviruses, namely Louping ill virus, Langat virus, and Powassan virus that also produce encephalitis on a smaller scale, are also presented. Here we describe the history and current epidemiological role of Omsk hemorrhagic fever virus and Kyasanur forest disease virus, two viruses that are genetically closely related to TBEV, but produce hemorrhagic fever instead of encephalitis, and provide possible explanations for these differences. The other viruses in the tick-borne flavivirus group are also included despite the fact that they do not play an essential epidemiologic role in humans. This chapter contains a brief history of vaccination against TBE including the trials with live attenuated vaccine and reviews the modern trends in development of vaccine virus strains.

I. Introduction

Tick-borne flaviviruses comprise one of the three major ecologic groups of viruses within the genus *Flavivirus*. The other two groups are mosquito-borne viruses and viruses for which there are no known vectors (NKV) (Calisher *et al.*, 1989, Porterfield, 1980). The previous name for the tick-borne virus group was the "tick-borne encephalitis (TBE) serocomplex." This referred mostly to those tick-borne viruses that were cross-reactive in serological tests, and/or were known to cause encephalitic disease in humans. Under the new classification scheme (Heinz *et al.*, 2000; also see Chapter 1), tick-transmitted flaviviruses comprise two distinct groups: mammalian and seabird virus groups. The most important recognized human pathogens in the mammalian group are tick-borne encephalitis virus (TBEV), Omsk hemorrhagic fever virus (OHFV), Powassan virus (POWV), Langat virus (LGTV), Kyasanur Forest disease virus (KFDV), and Louping ill virus (LIV), which previously comprised the group "TBE serocomplex." Other antigenically related mammalian tick-borne species (or subtypes) not known to cause human disease include Royal Farm virus (RFV), Karshi virus (KSIV), Gadgets Gully virus (GGYV), and Kadam virus (KADV), although Kadam virus is more closely related to seabird viruses (see later). The viruses associated primarily with seabirds are Tyuleniy virus (TYUV), Saumarez Reef virus (SREV), and

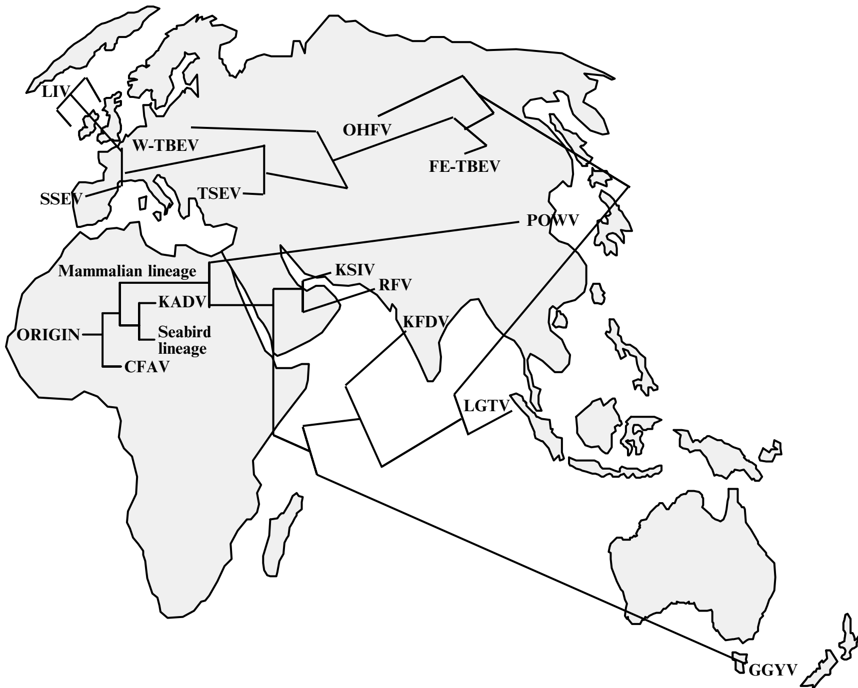

FIG 1. Evolution/dispersal of mammalian tick-borne flaviviruses.

Meaban virus (MEAV). Geographical location and phylogenetic relationships of these viruses are presented in Figs. 1 and 2.

Tick-borne encephalitis virus, which includes three subtypes, European, Far Eastern, and Siberian, plays the most important role in human epidemiology, particularly in the old "Eastern bloc" countries, producing a wide range of diseases including subclinical infections, biphasic fever, encephalitis, and chronic disease. In general, the other viruses in the mammalian tick-borne virus group may cause encephalitis or hemorrhagic fever but they do not result in major epidemic outbreaks. The seabird tick-borne virus group has not been shown to induce disease in humans or in seabirds and the reasons why these relatively closely related viruses differ so significantly in this particular characteristic have not been investigated. However, human contact with seabirds is limited.

All of these viruses survive and circulate in the natural environment, some of them forming epidemic foci of TBE. Long-term survival of the virus occurs mainly in ticks, which remain infected throughout

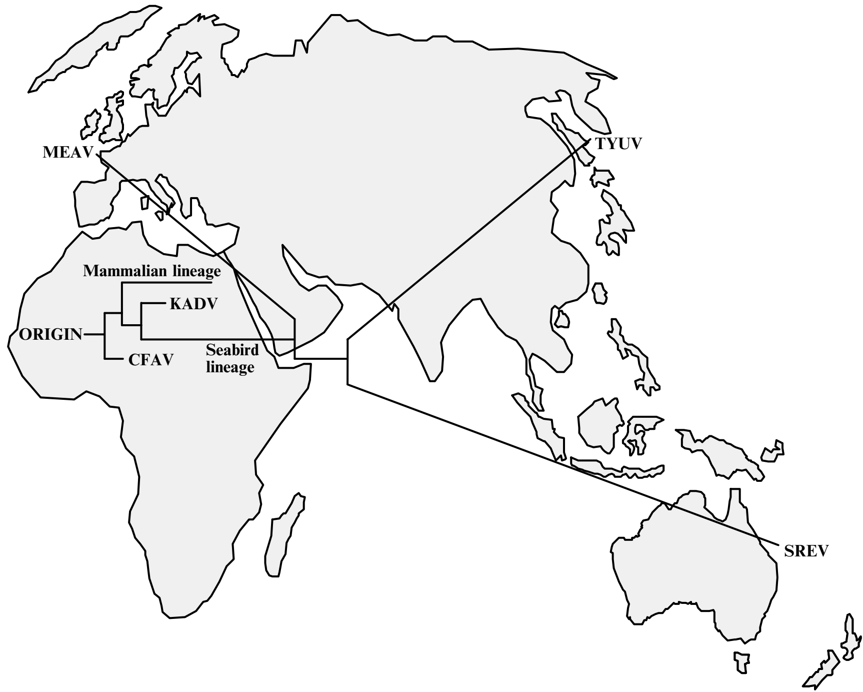

FIG 2. Evolution/dispersal of seabird tick-borne flaviviruses.

their life cycle and transmit virus to uninfected ticks during co-feeding on small wild rodents (Labuda *et al.*, 1996). The viruses have adapted to the physiologic and behavioral characteristics of ticks, particularly with regard to blood-feeding, blood-meal digestion, and moulting (Nuttall *et al.*, 1994); this is described in detail in other sections.

In this chapter we will cover the general epidemiologic characteristics of all the recognized tick-borne flaviviruses but because TBEV is the most important human pathogen within this group, we shall give particular emphasis to it.

II. TICK-BORNE ENCEPHALITIS VIRUS, A MAJOR HUMAN PATHOGEN

A. General Features and Incidence

Tick-borne encephalitis virus, previously known as Russian spring and summer encephalitis (RSSE) virus, was discovered in 1937 during an expedition in Far-East Russia led by Lev Zilber searching for the

etiologic agent of acute encephalitis. For many years, TBEV had been known to cause significant human morbidity and mortality in Siberia and Far East Russia during the spring and summer. Closely related virus strains are also distributed across Central and Western Europe but the frequency of disease association following infection is less. Although it is now known that the incidence of TBE varies from year to year over the different geographic regions of Russia (Korenberg and Kovalevskii, 1999), the Pre-Ural and Ural region as well as Siberia have the highest records of hospitalized cases (Korenberg and Kovalevskii, 1999; Zlobin and Gorin, 1996). During the 1950s and 1960s, the highest TBE occurrence was seen in forest workers, reaching 700–1200 cases annually. In the 1990s, following "Perestroika," the incidence of TBE increased (Korenberg and Kovalevskii, 1999; Zlobin and Gorin, 1996), with up to 11,000 recorded cases per year arising amongst urban dwellers who became infected when they visited the local forests. Previously, these people would have been immunized as a matter of routine but as the medical infrastructure deteriorated following "Perestroika," fewer people received immunization against TBE and the use of pesticides also ceased. Although the disease can affect people of all ages the highest incidence occurs amongst the most active groups (i.e., 17- to 40-year-olds) (Zlobin and Gorin, 1996). Infections arise following the bite of an infected tick. Surprisingly, serologic surveys suggest that more than 70–95% of TBEV human infections are subclinical (Pogodina *et al.*, 1986; Shapoval, 1976, 1977), indicating frequent exposure to infected ticks.

The incidence of clinically apparent forms of disease is dependent on several factors. First, a key determinant is the number of exposures to infected ticks. According to Shapoval (Shapoval, 1976), up to 45% of the local population in an endemic area receives at least one tick bite per epidemic season. Not surprisingly, the incidence of TBE correlates with the frequency of forest visits in endemic regions. It was established that during 10 visits to the forest each person on average is bitten twice. Although attached ticks are usually detected and removed, approximately 10% of attached ticks remained unnoticed for more than a few hours (Korenberg and Kovalevskii Yu, 1995). This may contribute to the incidence of infection because even a brief attachment of infected ticks can result in the development of TBE (Alekseev and Chunikhin, 1990).

The second factor associated with the incidence of TBE, infection prevalence in ticks, varies in different years and different regions over a range of 4–39% (Korenberg and Kovalevskii, 1999; Korenberg and Kovalevskii Yu, 1995; Korenberg *et al.*, 2001; Leonova, 1997). In an

endemic region, approximately 2.4% of the local population were estimated to come into contact with infected ticks over the epidemic season (Korenberg and Kovalevskii Yu, 1995).

The concentration of infectious virus in ticks, the third factor affecting TBE incidence, also varies. For example, in some regions in Far East Russia, approximately 61% of ticks contained specific antigen in low concentrations and approximately 17% in high concentrations (Leonova, 1997). Most people (approximately 60%) receive bites from ticks carrying low doses of virus and only 15% from highly infected ticks (Korenberg and Kovalevskii, 1999). Taking all these factors into account, it was estimated that one clinical case must occur for every 100 people bitten by ticks in endemic regions. These calculations correlated well with the fact that TBE develops in 1.4% of people who are bitten by a tick (Leonova, 1997).

In Russia, fluctuations in density of ticks in natural foci over the seasons are relatively small. The levels of maximum and minimum tick mass do not differ greatly over a period of years and do not correlate with infection rate in humans. Nevertheless the distribution of ticks within a natural focus is not uniform. Local zones, where tick density is much higher than in the surrounding areas, have been identified and designated nuclei of natural foci. The nuclei provide the most intensive circulation of virus and the highest incidence of TBE resulting from a tick bite. Therefore, although different factors contribute to the threat of infection when humans are exposed to infected ticks, the most important is the abundance of ticks containing a high dose of infectious TBEV (reviewed in Korenberg and Kovalevskii, 1999).

A fourth factor, related to the severity of TBE, is largely dependent on the virulence of TBEV strains, and this will be discussed in more detail later.

Disease incidence reaches maximal levels between May and July when ticks are most active in their search for a blood meal (Smorodintsev and Dubov, 1986; Zlobin and Gorin, 1996). The conventional belief is that ticks become infected when they feed on viremic hosts and this has been shown to be true in many laboratory experiments. However, it was realized some time ago that this is an oversimplification because efficient virus transmission can occur between ticks co-feeding on animals even if they are non-viremic (Labuda and Randolph, 1999; Labuda *et al.*, 1996). Indeed, infected ticks have been collected from wildlife species that do not develop detectable viremia (Jones *et al.*, 1997). Moreover, there is now evidence that this co-incident co-feeding transmission is an important factor in the maintenance of tick-borne

flaviviruses in the natural environment (Gould *et al.*, 2001; Randolph and Storey, 1999; Randolph *et al.*, 1999).

There have been extensive studies on TBEV in Russia where as many as 6000 articles and 50 monographs have been published (Korenberg and Kovalevskii, 1999). Following the isolation and antigenic identification of TBEV, efforts were made to identify and understand the so-called "genetic markers" of virus virulence. Although some properties of the viruses seemed to be associated with virulence, there has never been a satisfactory explanation for why apparently similar viruses produce different diseases. An understanding of these questions is relevant to the design and development of an appropriate strategy to control the disease. Below we highlight some of the research approaches that have been developed to identify the factors that may contribute to the virulence or pathogenic characteristics of TBEV.

B. Clinical Manifestations of Tick-Borne Encephalitis Virus Infection

Asymptomatic infections constitute approximately 70–95% of all TBEV infections (Pogodina *et al.*, 1986; Shapoval, 1976, 1977). When disease occurs, a variety of manifestations have been described. These can be summarized as follows.

i. Mild or moderate fever with complete recovery of patients.
ii. Subacute encephalitis with nearly complete recovery or residual symptoms that may or may not disappear over a long time.
iii. Severe encephalitis associated with irreversible damage to the central nervous system (CNS) resulting in disability or death.
iv. Russian scientists have also described a slow, progressive form of TBE that accounts for 2–5% of all cases with up to 8–13% in some regions (Shapoval, 1976). It is characterized by a long incubation period, sometimes taking years after the bite of the infectious tick before encephalitis appears. The condition then slowly deteriorates over a period of months or years eventually resulting in severe disability or death. The etiology of such chronic forms of TBE, in both sero-positive and sero-negative patients, was confirmed by virus isolation following the onset of symptoms (Levina and Pogodina, 1988; Pogodina *et al.*, 1986; Shapoval, 1976).

The incidence of different forms of TBE varies in different regions. For example, in Siberia approximately 80% of TBE infections that result in illness present as a mild or moderate fever without neurologic sequelae although hospitalization and special medical care are frequently required. Paralytic forms comprise approximately 7–8% and Kozshevnikov's

epilepsy (considered as one of the forms of chronic TBE) approximately 4–5% (Zlobin and Gorin, 1996). Approximately 7% of patients die following acute encephalitis, but the proportion of fatalities varies in different regions and the possible reasons for this will be considered below.

In view of the variety of clinical manifestations, it has frequently been suggested that there may be more than one variant of TBEV circulating in endemic regions. Nevertheless, because of the serological similarity of different virus isolates, for a long time the same virus was believed to produce tick-associated encephalitis over the whole of Europe and Asia. Indeed subsequent research into the pathogenic properties of different virus strains, in conjunction with their phylogenetic analysis, has demonstrated that the same virus species, namely TBEV, circulates across Eurasia, but at least three different virus subtypes (Heinz *et al.*, 2000; see subsequent section) are probably responsible for the different illnesses observed in man.

C. Geographic Distribution and Antigenic Similarity of Tick-Borne Encephalitis Virus

Classical tick-borne encephalitis is contracted by humans in or near forested areas across the northern hemisphere, where ticks inhabit the moist undergrowth. Small and large wild animals roam these areas and provide a food source (i.e., a blood meal) for the ticks. It is estimated that these conditions create at least 20,000–30,000 natural foci of tick-borne encephalitis from Western Europe, across Asia to Japan (Korenberg and Kovalevskii, 1999). Indeed for many years it was known that TBE viruses isolated from wide geographic areas were antigenically very closely related, and this implied that they might all represent variants of a single virus. Initially the existence of two different pathogenic variants of virus, central European encephalitis (CEE) and Russian spring and summer encephalitis (RSSE), was recognized (Chumakov *et al.*, 1944), later confirmed by serologic tests (Clarke, 1960). Subsequently, nucleotide sequencing (Mandl *et al.*, 1989; Pletnev *et al.*, 1990; Safronov *et al.*, 1991; Wallner *et al.*, 1996) resolved the problem of TBEV identity demonstrating close similarity between the CEE and RSSE virus genome (95% of protein homology).

Such genetic conservation appears to contradict the fact that TBEV is an RNA virus, and therefore should be prone to the rapid accumulation of mutations due to the high error rate of RNA-dependent RNA polymerases (Wells *et al.*, 2001). However, all the viruses within the mammalian tick-borne virus group have evolved as a complex of genetically very closely related viruses despite their wide distribution across

Eurasia. The explanation for this can be accounted for by a variety of factors. First, while virus undoubtedly replicates in the ticks, it never reaches high titers, thus reducing the likelihood of variation due to polymerase errors. Second, total virus turnover is relatively low in ticks, compared with the mosquito-borne flaviviruses because of the prolonged life cycle of the tick. *Ixodes* spp. feed only three times during their life cycle, first as larvae, which then emerge as nymphs. Months or years later, the nymphs feed and then emerge as adults. Again, months or years later, the adults feed and lay thousands of eggs, completing the life cycle. If larvae become infected, the descendant nymphs and adults remain infected throughout the rest of the life cycle and in some cases the virus is transmitted vertically to the next generation. Because this entire process may take up to 5 years, virus replication is severely constrained by the tick life cycle. Third, virus perpetuation occurs through co-feeding transmission (Gould *et al.*, 2001; Jones *et al.*, 1997; Labuda *et al.*, 1996), i.e., delivery of virus from infected to noninfected ticks when they co-feed at the same site on the skin of the mammalian host (discussed in Section II,E).

The major additional factor that determines the close genetic relationship of these viruses across a wide biogeographic area is the necessity for simultaneous co-feeding of uninfected larvae and infected nymphs on the local skin site of the mammalian host. Using Global Information Systems, it was demonstrated that the chances of this larval-nymphal synchrony depend largely on the climatic conditions (temperature, vegetation, moisture) within defined geographic limits, i.e., north and south (Randolph, 2000). If there is a rapid fall in ground-level temperatures, from August to October in the previous year, this means that large numbers of unfed larvae have to survive the winter in quiescence, from which they emerge synchronously with nymphs in the spring. Moreover, there must be high humidity to ensure a high ratio of co-feeding larvae. These conditions may not be satisfied every year, and this places a major constraint on virus transmission, rate of virus accumulation, and consequently, evolutionary selection pressure.

D. Association of Pathogenesis with Tick-Borne Encephalitis Virus Subtype

Despite their antigenic similarity, it is now recognized that TBEV isolated in western Europe (European subtype, previously CEE) are less pathogenic for humans than many of those isolated in Far East Russia (Far Eastern subtype, previously RSSE), although the complete

range of clinical manifestations may be observed following infection with either type (Burke and Monath, 2001). A third subtype of TBE virus was also proposed, based on clinical signs in humans and geographical location, previously called west-Siberian TBE (Siberian TBEV subtype according to new classification). Antigenic analysis confirmed the differentiation of west-Siberian TBE from CEE and RSSE viruses (Pogodina *et al.*, 1981a). Subsequent sequencing analysis completed this differentiation (Ecker *et al.*, 1999; Gritsun *et al.*, 1993a, 1997; Wallner *et al.*, 1995) and defined Siberian TBEV as a third subtype within the TBEV species (Heinz *et al.*, 2000).

Human infection with typical RSSE-like virus (Far Eastern subtype) results in the most severe form of CNS disorder with a tendency for focal meningoencephalitis or polyencephalitis to develop, accompanied by loss of consciousness and prolonged feelings of fatigue during recovery. In the most severe forms, there is major damage to neurons in different parts of the brain and spinal cord. Case-fatality rates of 20–40% have been recorded following outbreaks of RSSE in some years. During these epidemics, the slow progressive form of TBE is relatively rare.

In contrast, TBE viruses isolated in the west-Siberian region of Russia (Siberian subtype) are associated with a less severe acute period and a high prevalence of the non-paralytic febrile form of encephalitis. Case fatality rates rarely exceed 6–8%. Instead, there is a tendency for some patients to develop chronic TBE as defined in more detail in subsequent sections.

Encephalitis produced by CEE viruses (European subtype) is biphasic with fever during the first phase and neurological disorders of differing severity, during the second phase, which occurs for 20–30% of patients. In contrast to severe RSSE infections, those following infection by CEE are usually milder, mostly without sequelae; case fatality rates are often as low as 1–2% (reviewed in Votiakov *et al.*, 1978).

E. Association of Tick-Borne Encephalitis Virus Subtype with Tick and Rodent Species

Typical CEE and RSSE viruses are transmitted by different tick species, *Ixodes ricinus* and *Ixodes persulcatus*, respectively. It has also been shown that they have different sensitivities to dextran-sulphate implying that they could have different receptor specificity which could explain their different pathogenicities (Dzhivanian and Lashkevich, 1970; Dzhivanian *et al.*, 1974). However, these tests did not differentiate Siberian and RSSE type isolates, both of which are usually

transmitted by *I. persulcatus*. The possible association of disease pathogenicity (hemorrhagic fever or encephalitis) with vector species (*Aedes* spp. or *Culex* spp., respectively) has been noted for mosquito-transmitted flaviviruses, suggesting that selection by the arthropod could influence the pathogenic properties of the viruses for humans (Gaunt *et al.*, 2001).

Ixodes persulcatus is the most epidemiologically important vector for TBE viruses isolated from Siberia and the Far East comprising 80–97% of all tick species in many natural foci. Nevertheless other tick species (i.e., *Dermacentor pictus*, *D. silvarum*, and *Haemaphysalis concinna*) have also been associated with local TBE outbreaks in some areas, where *I. persulcatus* is not the predominant species (Zlobin and Gorin, 1996). TBEV has also been isolated sporadically from at least 15 other species of ticks and also many other parasites (fly, flea, lice, mosquitoes) (Smorodintsev and Dubov, 1986; Zlobin and Gorin, 1996), but their role in virus transmission to humans has not been demonstrated. Nevertheless, it is possible to suggest that even accidental virus multiplication in these other arthropods could result in the selection of new viruses with altered pathogenic characteristics for humans.

An analysis was performed to determine if there was any relationship between TBE incidence and different climatic and biological characteristics of natural foci in Far East Russia (Leonova, 1997). A good correlation of TBE incidence was found only with the level of *I. persulcatus* and *H. concinna*. A second and very interesting correlation was found between the severity of disease and the prevalence of *I. persulcatus* or *H. concinna*. In the years of high prevalence of *I. persulcatus*, the severe diseases and fatal cases were more frequent in comparison with those years in which *H. concinna* was a substantial part of the tick population. The presence of both tick species at the same time in one focus correlates with equal levels of severe and mild forms of TBE in the Far East. It was therefore proposed that different species of tick provide selection for high and low-virulence TBE strains (Leonova, 1997).

A similar correlation was found between the severity of disease and the rodent species, the hosts for ticks in natural foci. Grey-sided vole *Clethrionomys rufocanus* appeared to be a universal host for high- and low-virulence strains, whereas the common vole *Microtus arvalis* was only associated with low-virulence strains, implying a correlation between the feeding preferences of the ticks.

For many years, it was assumed that transmission of tick-borne viruses between mammalian hosts required the primary host to be

viremic and thus to provide the feeding tick with an infectious blood meal, subsequently transmitting the infection to a non-infected host for continuation of the transmission process. As discussed earlier, it is now known that efficient transmission of TBE complex viruses can occur between co-feeding ticks on a host which does not develop a detectable viremia (Labuda *et al.*, 1993). The local skin site of tick feeding was demonstrated as an important focus of viral replication early after TBE virus transmission by ticks. Cellular infiltration of tick feeding sites, and the migration of cells from such sites, may provide a vehicle for transmission between infected and uninfected cofeeding ticks. These data support the hypothesis that viremia is probably a product, rather than a prerequisite, of tick-borne virus transmission (Labuda *et al.*, 1996). It has also been shown that hosts having neutralizing antibodies to LIV or TBEV (and no detectable viremia) can still support virus transmission between infected and uninfected ticks feeding closely together on the same animal (Jones *et al.*, 1997; Labuda *et al.*, 1997). Thus, the virus may pass from infected to uninfected ticks with very little selective pressure being imposed on the virus by the mammalian host. Nevertheless naturally isolated escape mutants have been reported (Gao *et al.*, 1994), suggesting that selection might have occurred during feeding of infected ticks on immune animals (Labuda *et al.*, 1997). These observations have important epidemiologic implications relating both to the survival of TBE virus in animals and to the pathogenicity of the viruses for humans.

The influence of ticks on the biologic properties of TBE viruses has also been demonstrated in laboratory experiments. A TBE virus variant, adapted to *Hyalomma marginatum* in the laboratory, differed from the original virus by producing lower yields of infectious virus and an altered time course of virus release from infected cells. The released virions did not move toward the cathode in rocket immunoelectrophoresis; they showed reduced hemagglutinating activity, produced smaller plaques in PS cell culture, and showed a reduced level of replication in mouse brains. The acquired characteristics remained stable after three passages in mice. Following further re-adaptation to mice the virus recovered its original phenotype (Dzhivanian *et al.*, 1988, 1991). Similar results have also been reported for POWV (Khozinskaya *et al.*, 1985). In other independent experiments, serial passages of an uncloned TBEV in *I. ricinus* were accompanied by gradual reduction in virulence of the virus for mice and disappearance of hemagglutinating activity, suggesting that phenotypic changes, resulting from selection, had affected the receptor on the viral envelope protein.

Indeed sequencing of the envelope E glycoprotein showed one amino acid difference between the original and tick-adapted viruses (Labuda *et al.*, 1994).

A systematic comparison of flavivirus E protein sequences (Gritsun *et al.*, 1995) showed that the distribution of amino acid mutations in the virus receptor E protein does not occur randomly but is concentrated as 19 distinct hypervariable short amino acid clusters throughout the coding region. Most biologic characteristics of flaviviruses (i.e., antigenic differences, point mutations between vaccine and wild-type strains, altered tropisms, and neutralization escape mutants) grouped within these defined clusters. It was concluded that the clusters represent surface-orientated amino acid stretches, so-called "hot spots," for naturally occurring mutagenesis where random mutations do not destroy the critical elements of the E protein structure and are therefore important for virus survival. Possibly, the combination of amino acids in these clusters determines the "pathogenic face" of the particular virus isolates and can be considered as genetic markers of these viruses. The surface-orientated position of the clusters on the virion surface was subsequently confirmed by crystallographic analysis of the E protein (Rey *et al.*, 1995). Recently it was demonstrated that 12 random mutations that arose in the E protein during passage of TBEV in cell culture, mapped within the identified clusters (Mandl *et al.*, 2001). The mutation $E_{84} \rightarrow K$ that was associated with TBEV adaptation to *I. ricinus* (Labuda *et al.*, 1994) also mapped in one of these clusters and was identical to the amino acid substitution associated with adaptation of TBEV to BHK cells (Mandl *et al.*, 2001). Although not excluding the possibility that adaptation of TBEV to ticks might be accompanied by mutations in other genes, selection of tick-adapted variants appears to be associated with mutagenesis in the E protein clusters. This could define how flaviviruses with different pathogenic characteristics emerge.

F. Experimental Modeling of Tick-Borne Encephalitis Virus

There are many experimental data that demonstrate pathogenic differences between RSSE and CEE viruses and RSSE and Siberian TBE strains (Votiakov *et al.*, 1978).

Comparative analyses between CEE and RSSE viruses producing different forms of experimental TBE have been carried out on monkeys and sheep (Votiakov *et al.*, 1975, 1978). The results can be summarized as follows:

- Subcutaneous inoculation of these animals with typical CEE or RSSE isolates did not result in encephalitis.
- Following natural infection with TBEV, sheep became sick but without developing encephalitis.
- Following intracerebral inoculation of TBEV, both monkeys and sheep developed encephalitic symptoms although the disease produced by CEE virus was less severe and a lower proportion of animals developed encephalitis than was seen following infection with RSSE virus (Votiakov *et al.*, 1978).

Comparative pathologic and virologic analysis revealed differences in both the spread and extent of damage in sheep infected with the two viruses. RSSE virus directly infected and damaged neurons in the brain, resulting in severe encephalitis. In contrast, CEE virus initially did not replicate in or damage neuronal cells even after intracerebral inoculation of virus. Instead, the primary target of CEE virus was lymphoid tissue with subsequent re-appearance of the virus, 6–9 days after inoculation, in the brain (predominately in the cerebellum) of those animals that developed encephalitis. These observations have important implications for our understanding of TBEV pathogenesis.

A summary of quantitative comparisons revealing different degrees of neuronal damage during the course of TBE produced in sheep by either RSSE or CEE virus is presented in Table I (Votiakov *et al.*, 1978).

TABLE I
COMPARISON OF PATHOGENESIS OF CEE AND RSSE IN SHEEP

Type of observation	CEE	RSSE
Virus presence in CNS	Tendency to clear virus from CNS and lymphoid tissue	High virus titers in CNS until death
Damage to neurons	Resulting from secondary or inflammatory response of host in second phase of the disease	Primary and specific effect of virus infecting neurons as primary targets
Pathomorphologic changes in CNS	Predominantly mesenchymal infiltration	Primary degeneration of neurons
Maximum extent of CNS damage	Sub-acute encephalitis	Very rapid coma-like encephalitis
Fatality rate	12.5%	100%

CEE, Central European encephalitis; CNS, central nervous system; RSSE, Russian spring and summer encephalitis. From Votiakov *et al.*, 1978.

The data produced in monkeys and sheep correlated well with statistics for human infections and also supported the distinction of the TBEV as two groups, CEE-like and RSSE-like, that now are classified as two different TBEV subtypes. On the basis of this research, it was concluded that RSSE viruses frequently produce severe encephalitis with fatal outcome because they have a tropism for neurons of the brain and spinal cord, and rapid degeneration of neurons results from replication of the virus in these cells. In contrast CEE viruses do not produce severe infection in the neurons. Damage to the neurons occurs only in some animals and is a consequence of secondary inflammation arising from infection of glial cells (Votiakov *et al.*, 1978).

As discussed previously, a third group of TBE strains, the Siberian subtype, was identified before confirmation of its existence based on sequence data. This virus was isolated from both acute and chronically infected patients as well as ticks and rodents in Siberia. The prototype virus of this group, Aina, was differentiated serologically from both RSSE and CEE viruses (Pogodina *et al.*, 1981a). A significant etiologic role for Aina serotype viruses in both acute and chronic forms of TBE was supported by serologic analysis of patients analyzed months or years after the onset of the disease. Most of the viruses belonging to this group were isolated from *I. persulcatus* and a few were from *Dermacentor nuttalli*. Another well-characterised virus strain in this serogroup is Vasilchenko (Vs) (Frolova *et al.*, 1982a, 1987; Gritsun and Gould, 1998; Gritsun *et al.*, 1993a, 1997, 2001).

The difference in pathogenesis between Far Eastern (RSSE) and Siberian (Aina and Vs) subtypes of TBEV was demonstrated experimentally using Syrian hamsters. Intracerebral inoculation of hamsters with 10^5–10^7 LD_{50} resulted in different clinical manifestations that became the basis for classifying viruses into three groups: highly virulent, moderately virulent, and low virulence. Highly virulent viruses produced severe paralysis and fatal outcome in 67–100% of animals with an average survival time of 5–60 days. Moderately virulent viruses produced paresis in 29–58% of animals with average survival times between 86 and 210 days. Low virulence viruses were pathogenic for 5–17% of hamsters with an average survival time of 100–237 days. Impressively, the serologic tests showed that all highly virulent strains were closely related to the RSSE virus group, and the moderately and low virulence strains were serologically most closely related to the Siberian group (Pogodina *et al.*, 1986). Strains of TBEV isolated from patients with chronic TBE demonstrated moderate or low virulence characteristics in hamsters. A general feature of all these strains specified as Siberian subtypes was

their ability to produce slowly developing pathology and asymptomatic infections.

Monkeys have also been used to compare the pathological properties of TBEV isolated in Far East Russia and Siberia (Pogodina *et al.*, 1986). One hundred thirteen strains of TBE virus isolated from different geographical regions and from different sources (patients, ticks, rodents, goats' milk) were inoculated intracerebrally into monkeys. Most of the strains produced encephalitis with fatal outcome (76%), while 14% of animals developed encephalitis without fatal outcome (8% with full recovery and 5% with hyperkinesis). Ninety-eight percent of these animals remained healthy and 2% developed chronic encephalitis. Eight strains from 113 produced chronic TBE in monkeys; one was isolated from Far East Russia, four from Siberia, three from Karelia, and three from Northwest Russia. Considering the vast territorial distribution of viruses specified as Siberian subtype that were originally assigned only to Siberia (Mavtchoutko *et al.*, 2000), this could be considered as evidence that chronic forms of TBE are more frequently associated with the Siberian group of TBE viruses than with RSSE or CEE viruses.

G. Chronic Forms of Tick-Borne Encephalitis Virus

This section highlights different aspects of chronic TBE that have been studied and originally reviewed in Russian (Pogodina *et al.*, 1986; Shapoval, 1976). The ability to persist and produce chronic disease in humans and animals has been demonstrated for many tick-borne and mosquito-borne flaviviruses, including TBEV (Frolova *et al.*, 1982a), LIV (Shen, 1961; Zlotnik *et al.*, 1971), LGTV (Illavia and Webb, 1970; Zlotnik *et al.*, 1973), KFDV (Price, 1966), Japanese encephalitis virus (Mathur *et al.*, 1982, 1987; Pogodina *et al.*, 1996; Ravi *et al.*, 1993; Sharma *et al.*, 1991), and West Nile virus (WNV) (Pogodina *et al.*, 1983). There are two major manifestations of chronic TBE infections: those with and those without a preliminary acute period. Independently of this, the general feature of all chronic TBE infections is the prolonged slow progressive development of neurologic symptoms with or without fatal outcome. There are a wide variety of clinical pictures relating to chronic TBE infections in humans with variations in the incubation period and onset of disease, longevity of development of the symptoms of neurologic disorders, and the period of patient survival after the onset of disease. Clinical manifestations of chronic encephalitis are also different and include Kozshevnikov's epilepsy, progressive neuritis of the shoulder plexus, lateral sclerosis,

dispersed sclerosis, a Parkinson-like disease, and progressive muscle atrophy (Shapoval, 1976; Votiakov *et al.*, 1978). The etiologic association of these neurologic disorders with TBE virus was proved by regular isolation of virus from the blood of patients and the brain of fatal cases of chronic encephalitis (Pogodina *et al.*, 1986; Shapoval, 1976).

Initially, experimental evidence of the possible existence of chronic forms of TBE came from modelling chronic infections in monkeys (Fokina *et al.*, 1982; Frolova and Pogodina, 1984a; Pogodina *et al.*, 1981b, 1981c, 1981d). For these forms of disease the characteristics were a prolonged incubation period, progressive development of neurologic symptoms, followed by paralysis of the limbs or partial recovery of movement functions. Different strains of TBE virus isolated from different geographical regions and demonstrating different virulence characteristics in cell culture and white mice induced chronic TBE in monkeys. During chronic TBE and latent infection, virus could be isolated from different organs including the spleen, liver, lymph nodes, kidney, and different parts of the CNS (Pogodina *et al.*, 1986). In asymptomatic infection, TBE virus spread through the same organs of the host as reported for acute encephalitis (Fokina *et al.*, 1982). In the early stages of infection (3–19 days), virus was isolated mainly from the CNS. After 45 days, it was not possible to isolate virus from the CNS and later it could only be identified in the internal organs.

The Siberian isolate of TBE virus, Vs, was largely used to model chronic TBE in monkeys and Syrian hamsters. This virus was isolated in the Novosibirsk region in 1961 by Kvetkova from the blood of a patient with a non-paralytic form of TBE (Pogodina *et al.*, 1986). In monkeys, Vs produced a broad variety of clinical diseases including chronic forms that developed either after non-lethal encephalitis or after asymptomatic infection. The descendant of Vs virus, called Vs-383, was isolated on day 383 from an experimentally infected monkey that developed non-lethal encephalitis with stable paralysis of one of the front limbs (Malenko *et al.*, 1982). Subsequently, it was demonstrated that Vs-383 had become more virulent for Syrian hamsters (51% lethality compared with 5% for Vs virus).

Two groups of animals were inoculated either with Vs or Vs-383 and were monitored for 2 years (Frolova and Pogodina, 1984b; Frolova *et al.*, 1982a, 1982b). Four groups of viruses were isolated and characterized during this period of observation. The first group was isolated from animals with fatal encephalitis that developed shortly after infection. The second group was isolated from animals that developed symptoms of chronic neurologic disorder imitating the clinical picture

of patients with chronic progressive forms of TBE. The third group was collected from animals that remained healthy for a long period after inoculation and thus represented the asymptomatic infections. The fourth group was isolated from healthy animals that were inoculated 2 years after infection with different immunosuppressants such as adrenaline, cyclophosphamide, prednisolone or vincristine (Frolova and Pogodina, 1984b; Frolova *et al.*, 1982b). Each of these immunosuppressants activated the replication of latent TBE viruses, causing sickness in the hamsters approximately 2 years after the original virus inoculation. These findings provided evidence that TBE virus persists in apparently healthy animals.

Another Siberian strain of TBE virus, designated Zausaev, was isolated from the medulla oblongata of a patient who had died from progressive infectious encephalitis (T. V. Frolova, personal communication). The first mild symptoms of movement disorder had appeared 10 years after the tick bite. The symptoms of progressive encephalitis then intensified during the next 2 years and despite medical treatment, the patient died from acute encephalitis. Zausaev virus was not pathogenic for Syrian hamsters. The complete sequence of Zausaev virus demonstrated that it belongs to the Siberian subtype of TBE viruses. Nevertheless, several unique non-conserved amino acid substitutions were identified that might have appeared during the development of long-term persistence in the patient and may define the virus' pathogenicity (manuscript in preparation).

H. Biphasic Milk Fever

After World War II, an unusual outbreak of TBE was reported in the western part of European Russia. Epidemiologic surveys established that very few of these TBE cases were associated with tick-bites. Although the virus was identified as TBEV, the course of disease was always biphasic while during classical TBE the biphasic form was observed in only 20–30% of cases (Shapoval, 1961). Meningoencephalitis, with slightly developed meningeal and parenchymal symptoms, was exclusive to milk fever, whereas the conventional clinical picture of tick-associated TBE is aseptic meningitis, meningoencephalitis, meningoencephalomyelitis, or meningoencephaloradiculitis. An unusual feature was whole families contracting TBE during epidemics (Shapoval, 1989). Eventually it was established that this form of disease was associated with the consumption of goat's milk that was drunk as a substitute for cow's milk immediately following World War II. Tick-borne encephalitis virus was isolated from the milk of

goats for 5–25 days following experimental infection, and the infectivity survived in different unpasteurized milk products (yogurt, cheese, butter) (Shapoval, 1977). Later, outbreaks of biphasic milk fever were reported in Siberia and were associated with local endemic strains of TBEV (Pogodina *et al.*, 1986; Shapoval, 1961). Biphasic milk fever is considered to be an epidemiologic variant of TBE rather than a disease etiologically associated with a particular strain of TBEV. The differences in clinical manifestations between TBE contracted by tick-bite or by the alimentary tract are explained by differences in the immunologic response that depend on the route of virus penetration and the initial concentration of virus.

The oral route of infection is believed to be mediated by multiplication of TBEV in gastric or intestinal tissues. Experimentally, the infectivity of TBEV is stable for up to 2 hours in normal human gastric juice at pH 1.49–1.80 and in gastric juice with reduced acidity (pH 1.87–2.21) (Pogodina, 1958). The consumed milk reaches the duodenum within minutes and after 1.5–2 hours there is no milk in the stomach. Hydrochloric acid is secreted in the stomach between 45 and 60 minutes after consumption of the milk and therefore there is a period when the virus has the opportunity to enter the cells of the human digestive tract without acidic inactivation. In laboratory experiments, mice became infected after they were fed orally with TBEV (Pogodina, 1960) or Japanese encephalitis virus, a mosquito-borne flavivirus (Ramakrishna *et al.*, 1999). Additionally, grouse chicks developed lethal encephalitis following feedings orally with ticks infected with LIV (unpublished observations). In this case the ticks were swallowed intact; therefore, these experiments suggest that the portal of entry is via the gastric rather than the oropharyngeal route. However, the aerosol route of infection with flaviviruses has been demonstrated (Scherer *et al.*, 1980), and therefore it is not possible to exclude the possibility that the oropharynx could also provide a route of virus entry.

III. Other Encephalitic Tick-Transmitted Flaviviruses

There are three other tick-borne flavivirus species that cause encephalitis in humans and/or animals. These are Louping ill virus (LIV), Powassan virus (POWV) and Langat virus (LGTV). Although they are antigenically related, they can be distinguished serologically from the TBE viruses described previously and from each other (Calisher, 1988; Porterfield, 1975). Subsequent phylogenetic analysis has essentially proven the validity of the serologic classification (Gould *et al.*, 2001)

and enabled predictions that the tick-borne flavivirus lineage diverged from the mosquito-borne flaviviruses less than 5000 years ago (Zanotto *et al.*, 1995, 1996). In general these viruses do not produce significant epidemic outbreaks and therefore, compared with the TBEV, they have attracted less attention. However, understanding the differences and similarities between these viruses and TBEV may help to define the major factors that determine virus neurovirulence.

A. Louping Ill Virus

Louping ill virus (LIV) is the only flavivirus that has been identified in the United Kingdom. The disease louping ill is recognized predominantly as a fatal encephalomyelitis of sheep that become infected when they are bitten by LIV-infected tick. The disease is seen on the uplands of Scotland, northern England, north Wales and southwest England, where the thick mat of vegetation provides a suitably moist environment for long-term survival of the LIV tick vector *Ixodes ricinus*. In Ireland, the disease is often seen on the lower slopes of farms because of the increased moisture content of the grasses and therefore, infection among horses, cows, and pigs is not uncommon. Red grouse (*Lagopus lagopus scoticus*) are also highly susceptible to LIV, developing a fatal encephalitis with rapid onset. Mild wet winters provide good conditions for increased numbers of ticks, and under these conditions, the grouse populations in Scotland and north Wales are usually severely threatened by LIV (Reid and Boyce, 1974). Roe deer that also roam the highlands provide an excellent amplification host for tick numbers while being insusceptible to LIV. Separation of sheep and deer is therefore advisable on moorlands. In contrast to the subclinical infection in sheep exposed to TBEV-infected ticks, LIV-infected young non-immune sheep develop a biphasic illness (Doherty and Vantsis, 1973; Reid and Doherty, 1971a, 1971b; Reid *et al.*, 1972). The first phase is characterized by fever and weakness, followed by a second neurologic phase with prominent cerebral ataxia, hyperexcitability, and progressive paralysis, the main damage being to the cerebellum. The virus can be transmitted from infected to uninfected ticks feeding in proximity on blue mountain hares (*Lepus timidus*) in the absence of viremia (Jones *et al.*, 1997). This observation may partly explain how LIV still persists in the natural environment when introduced sheep have been specifically immunized against the virus and also treated with acaricides.

A similar disease in sheep, reported in Norway, is caused by LIV-infected sheep/ticks introduced from mainland Britain (Gao *et al.*,

1993b). Sheep and goat encephalomyelitis has also been reported in Spain (SSE), Greece (GGE), and Turkey (TSE), and sequencing analysis confirms its etiologic association with LIV (Gao *et al.*, 1993a; Marin *et al.*, 1995a). The viruses that cause these infections are genetically distinct from LIV (Gould *et al.*, 2001; McGuire *et al.*, 1998). They probably emerged on the hillsides of Greece, Turkey, and Spain when sheep were introduced near to wooded areas where TBE virus was present. Some of the TBE-infected ticks probably fed on sheep/goats that roamed the edges of the forests and transferred the virus from the forest environment to the hillsides, whereupon variants more suitable for these animals were selectively enriched.

The clinical picture for humans infected with LIV is very similar to that produced by European subtypes of TBEV. The first phase of disease is characterized by fever, lasting 2–11 days, followed by remission lasting 5–6 days, and then the reappearance of fever and meningoencephalitis lasting 4–10 days. There have been very few reported cases of encephalitis in humans, mostly among laboratory personnel (Davidson *et al.*, 1991). Although the virus is potentially a serious threat, human exposure to LIV is rare and probably most often results in subclinical infections.

Systematic analysis of large numbers of LIV strains demonstrated sequence variation (Gao *et al.*, 1993a, 1993b; Venugopal *et al.*, 1992). Evolutionary origins and dispersal patterns of LIV strains in the British Isles have been described (McGuire *et al.*, 1998).

B. Langat Virus

Langat virus (LGTV) was isolated in Malaysia and neighboring Thailand from pools of *Ixodes granulatus* and *Haemaphysalis* ticks (Smith, 1956). The virus infects rodents, particularly ground rats (*Rattus muelleri validus*) and the noisy rat or long-tailed rat (*Rattus sabanus vociferans*). It is not known to cause overt disease in rodents in their natural environment, but encephalitis develops in young laboratory mice inoculated intracerebrally with the virus. Although there are no registered cases of human disease associated with this virus, specific antibodies against LGTV were found in the sera of local people.

The virus is completely avirulent for adult mice following subcutaneous or intraperitoneal inoculation and for nonhuman primates following intracerebral inoculation. In the few cases that arose following vaccination with live attenuated LGTV-based vaccines (discussed

subsequently), the encephalitis was similar to that seen following infection with typical European strains of TBEV.

Significant disease among wildlife species has not been reported, implying that LGTV has been established in the forests of Malaysia for a considerable time and has not yet been disturbed either directly by humans or indirectly through the introduction of non-indigenous species. In evolutionary terms, LGTV forms part of the cline described elsewhere in this book and, according to phylogenetic analysis, diverged before the first appearance of the TBEV described above. Both antigenic and phylogenetic analysis distinguish LGTV from other tick-borne flaviviruses (Calisher, 1988; Marin *et al.*, 1995b; Porterfield, 1975).

C. Powassan Virus

Powassan virus (POWV) circulates in South Dakota, the Eastern and Western United States, Canada, and Far Eastern Russia (Isachkova *et al.*, 1978; Johnson, 1987; Leonova *et al.*, 1980; Lvov *et al.*, 1974) and is readily differentiated from the other tick-transmitted virus species by serologic tests. It was first isolated in 1958 from the brain of a 5-year-old boy who died from encephalitis in Powassan, Ontario, Canada (McLean and Donahue, 1959). In North America, POWV causes severe encephalitis in humans with a high incidence of neurologic sequelae and up to 60% case-fatality rate. In laboratory experiments, POWV also produces lethal encephalitis in primates (Frolova *et al.*, 1981, 1985). In Far Eastern Russia POWV infections were described as milder than those produced by TBEV and some differences were noted between the lesions in the CNS caused by POWV and TBEV (Leonova *et al.*, 1991). Moreover, independent research provided evidence of latent infection with POWV in humans, domestic pets (cats and dogs), and local rodents (Leonova *et al.*, 1987b; Mandy *et al.*, 1979). Latent infection and milk-borne transmission of POWV in goats has been demonstrated experimentally (Woodall and Roz, 1977).

Encephalitis due to POWV is seen only sporadically in Canada, with 27 reported cases in North America since 1958 (www.hc-sc.gc.ca/pphb-dgspsp/msds-ftss/msds121e.html). In Russia, POWV was first isolated in 1973 and only 14 cases were recorded during the next 16 years (Leonova *et al.*, 1987b). Nevertheless, in Russia POWV co-circulates with TBEV in the same environment and the real incidence of encephalitis associated with POWV might be masked by the encephalitis produced by TBEV or even mixed infections which have been shown to occur (Leonova *et al.*, 1987b). For example, between 1976 and

1978 among 117 patients with acute TBE, antibodies to TBEV were detected in 69%, to POWV in 4%, and to both viruses in 4% of cases. Moreover, antibodies to TBEV (9%), POWV (1.6%), and both viruses (one case among 440) were detected in healthy people bitten by ticks. Among 74 patients with the chronic progressive form of TBE, neutralizing antibodies to TBEV were present in 25%, to POWV in 7%, and to both viruses in 7% of cases (Leonova *et al.*, 1980). Although it was not reported, the other 61% did not have detectable antibodies to either virus. These data indirectly support the observations that in endemic regions, seronegative cases of TBE are not particularly rare.

Thus, there are differences in the epidemiology of POWV between North America and Asia, with higher incidence but milder infections in Far Eastern Russia in comparison with those in North America. At present, there is no satisfactory explanation for this but the differences might reflect the peculiarities of POWV strains associated with the ecologic conditions on different continents. In the natural environment in Canada, the most important enzootic vector, *Ixodes cookei*, feeds mainly on groundhogs (*Marmota monax*), attacking other hosts including humans only rarely. POWV has also been isolated from other tick species including *Ixodes angustus*, which often bite humans and cats, and *Ixodes scapularis* and *Dermacentor variabilis*, which frequently bite humans and dogs (Mandy *et al.*, 1979).

In Russia, POWV has been isolated from *I. persulcatus*, *H. neumanni*, *H. consinna*, and *D. silvarum* (Krugliak and Leonova, 1989; Lvov *et al.*, 1974) and replication in different tick species is believed to be a selection factor for different POWV strains. In laboratory experiments, POWV has been shown to be transmitted more effectively from infected white mice to *I. persulcatus* and *H. consinna* (at a lower level of viremia), than to *D. silvarum*. Nevertheless, in the environment POWV was isolated predominantly from *D. silvarum* possibly because these ticks have a shorter life cycle feeding twice annually, as larvae and nymphs, giving them a potential advantage for virus transmission (Krugliak and Leonova, 1989). An alternative explanation could be that virus transfer is mediated more efficiently on *D. silvarum* by non-viremic co-feeding of infected and non-infected ticks at the local skin site of the host, as described previously.

Local rodents in Russia also probably play an important role in selection of different POWV variants. In the laboratory POWV has been shown to replicate more efficiently in wild rodents *A. peninsulae Thomas* and *C. rufocanus Sund* than in *A. agrarius Pall* or *M. fortis Butchen*, although POWV demonstrated lower reproduction levels in

all wild rodent species, when compared with TBEV (Leonova *et al.*, 1987a).

Phylogenetic analysis shows that POWV emerged as the most ancestral lineage of the mammalian tick-borne viruses (Zanotto *et al.*, 1995). Interestingly, the virus has also been isolated from mosquitoes, *Anopheles hyrcanus* and *Aedes togo* (Kislenko *et al.*, 1982). In laboratory experiments in *Aedes aegypti* and also mosquito cell cultures, virus could be recovered for the first 2 weeks following infection but subsequently no infectious virus was recovered. In contrast with other tick-borne flaviviruses, POWV has an extra amino acid in the envelope E protein sequence, D_{336}, that is shared with mosquito-borne flaviviruses (Gritsun *et al.*, 1995; Mandl *et al.*, 1993).

A genetically closely related virus known as deer tick virus (DTV) was recently isolated from the deer tick *Ixodes dammini* (Telford *et al.*, 1997). This virus is maintained in separate enzootic cycles from POWV. On the basis of nucleotide sequence and phylogenetic analysis, DTV is considered to be sufficiently different from POWV to be a distinct subtype (Ebel *et al.*, 2001). No human disease has been reported.

IV. Non-Encephalitic Mammalian Tick-Borne Flaviviruses

The viruses described in this section have not been studied in as much detail as the other viruses within the mammalian group of tick-borne flaviviruses because they are not associated with disease.

A. Karshi Virus

Karshi virus (KSIV) was isolated from the tick species *Ornithodorus papillipes* collected in 1972 from the burrow of a Great Gerbil in Uzbekistan (Lvov *et al.*, 1976). This virus cross-reacts with several tick-borne flaviviruses and surprisingly, in neutralization tests, it cross-reacts strongly with West Nile virus, a mosquito-borne virus. Nevertheless, phylogenetic analysis showed that KSIV is a member of the mammalian tick-borne viruses and, apart from Royal Farm virus, is most closely related to POWV and KFDV (Gaunt *et al.*, 2001; Gould *et al.*, 2001; Kuno *et al.*, 1998). There are no reports of human disease resulting from infection by KSIV, although its close phylogenetic relationship with KFDV and POWV suggests that this virus should be capable of causing disease in humans.

B. Royal Farm Virus

Royal Farm virus (RFV) virus was isolated from *Argas hermanni* nymphs in Kabul province, Afghanistan. These nymphs were collected in 1968. Antigenically the virus is most closely related to POWV and LGTV (Williams *et al*., 1972). This relationship is supported by phylogenetic analyses (Gaunt *et al*., 2001; Gould *et al*., 2001; Kuno *et al*., 1998). Royal Farm virus is now considered to be a subtype of KSIV (Heinz *et al*., 2000). In common with KSIV, no human disease has been associated with RFV but it is reasonable to assume that if the virus was transmitted to a human host, it might cause an encephalitic or hemorrhagic disease.

C. Gadgets Gully Virus

Gadgets Gully virus (GGYV) was isolated from Macquarie Island in the Southern Ocean (St. George *et al*., 1985) from seabird-associated ticks *Ixodes uriae*. Nevertheless, sequence analysis indicates that it is genetically more closely related to mammalian tick-borne flaviviruses than the seabird flavivirus group and therefore it was classified as a species within the mammalian tick-borne group (Gould *et al*., 2001; Kuno *et al*., 1998). Nevertheless it represents an early lineage in the evolution of the mammalian tick-borne virus group and therefore probably represents an evolutionary link between the seabird tick-borne virus group and the mammalian tick-borne virus group.

V. "Hemorrhagic" Tick-Borne Viruses

There are three recognized viruses within the mammalian tick-borne virus group that cause hemorrhagic fever: Omsk hemorrhagic fever virus (OHFV), Kyasanur Forest disease virus (KFDV), and Alkhurma virus (ALKV), which is closely related to KFDV (Charrel *et al*., 2001) and will probably be designated a subtype of this virus in the future. These viruses form part of the evolutionary cline that was described as a characteristic of the TBE complex viruses (Zanotto *et al*., 1995). Although they are best known for their capacity to produce hemorrhagic disease in humans, sporadic cases of encephalitis have also been attributed to these viruses. Currently, the genetic factors that determine virus association with hemorrhagic manifestations rather than encephalitic disease are unknown although there is accumulating evidence from other hemorrhagic flaviviruses that sequences in the non-structural regions of their genomes may play an important role.

A. Omsk Hemorrhagic Fever Virus

The first descriptions of Omsk hemorrhagic fever were reported in 1941–1944 but it was not recognized as a unique disease associated with a virus closely related to TBEV until 1947 (Belov *et al.*, 1995; Lvov, 1988). Subsequent sequence analysis confirmed the close phylogenetic relationships of OHFV with other tick-borne flaviviruses (Gritsun *et al.*, 1993b). The epidemic foci characteristically occur in the Omsk and Novosibirsk regions of West Siberia, associated with the distinctive landscape of these regions (i.e., the forest-steppe and nearby lakes). There are also recent reports of emerging OHF-like clinical manifestations in the Ukraine, Smolensk, North Kazakhstan, and Orenburg, although this information requires more confirmation (Busygin, 2000).

Sporadic human epidemics of hemorrhagic fever were associated with epizootics in muskrats (*Ondatra zibethica*) that were reintroduced into Omsk region in 1928 from Canada to replace the extinct species. Before the introduction of muskrats, which are highly susceptible to the disease, OHF would probably have been very rare in humans and it is unlikely that the virus would have been identified by virologists. On the other hand, the indigenous small mammalian species (i.e., rats, voles, mice, hamsters, polecats, birds) are much less susceptible to infection by OHFV (Kharitonova and Leonov, 1985). Muskrats were hunted by the local farmers who then became infected when they skinned the animals. It is presumed that the farmers transmitted the virus to their close relatives possibly by direct contact.

The clinical manifestation of human infections is different from other TBE infections. The onset is marked by fever, headache, myalgia, and cough, which progress to bradycardia, dehydration, hypotension, and gastrointestinal symptoms. The most marked pathologic signs of the disease are focal visceral hemorrhages in the mucosa of the nose, gums, uterus, and lungs. Clinical symptoms also include diffuse encephalitis, which disappears during the recovery period. Convalescence is usually uneventful without residual effects; fatal cases are also registered, but rarely (0.5–3%; Kharitonova and Leonov, 1985).

The history of OHF can be subdivided into two periods, one between 1946 and 1958, which commenced with mass epidemics between 1946 and 1952 turning into sporadic cases; and a second period from 1958–1988 during which no cases of OHF were formally registered (Busygin, 2000).

According to official data, there were 972 cases between 1946 and 1958, but this does not reflect the true incidence because physicians

encountered only severe cases. Milder cases were not examined and it is assumed that altogether there were at least 1344 cases (Busygin, 2000), implying that OHFV could cause mild or asymptomatic (subclinical) infections.

The disease emerged as different epidemic patterns including large epidemic outbreaks, localized outbreaks, and sporadic cases, among 133 different residential areas. Ninety-seven percent of the cases were recorded in the northern forest-steppe regions and 3% in the south. In northern regions, *Dermacentor reticulatus* was the dominating tick species, whereas in the southern region the vector was *D. marginatus*. The incidence of OHF was highest among the 20- to 40-year-old age group and children younger than 15 comprised one third of all cases. The first cases emerged in April (1% of the annual total number of cases) and the last in October (1%) with a maximum incidence during May and June of 73% and a second peak during August and September of 21%.

Outbreaks of OHF that arose following contact between humans and infected muskrats normally occurred in late autumn, which was the peak period both for epidemic outbreaks among these animals and hunting. The other distinctive and interesting feature of these muskrat-associated outbreaks is the absence of the asymptomatic forms of disease that were observed in individuals infected through the bite of a tick. This is probably because the virulence of the virus increased during passage through the muskrats.

During 1960–1970, the general opinion amongst the scientific community in Russia was that the foci of OHF were disappearing, but later observations demonstrated that it was not that simple. Serological surveys and studies of tick infectivity established that OHFV did not disappear but that its distribution had changed. It was shown that the density and infectivity of *D. reticulatus* and its main host, the narrow-skulled vole (*Microtus gregalis Pall*) had decreased, thereby reducing their contribution to epidemics and enzootics. Further research into the ecology and epidemiology of OHFV led to the conclusion that there are two types of "pseudo-foci" (Busygin, 2000), one described as "active" with about 35% of seropositive people including young children, and another described as "potential" with low levels (about 15%) of immunity and no seropositive children under 14.

The active pseudo-foci are located around "living" lakes with rich vegetation and an abundance of small animals that attract muskrats, which support active virus circulation. The potential pseudo-foci typically are located far away from lakes or surround "non-living" lakes with water containing high mineral levels making them unsuitable habitats for muskrats (Busygin, 2000).

Seroepidemiologic data among local populations enabled scientists to predict the possibility of emerging OHF despite the absence of typical hemorrhagic disease. Indeed in 1970–1975, among 577 patients with fever of unknown etiology examined by physicians, OHF was diagnosed in 18% of people exposed to active pseudo-foci and 4% exposed to potential pseudo-foci. Therefore, in reality the disease did not disappear. It is now believed that OHF naturally occurs as a relatively mild form of disease except under circumstances when the muskrat supports virus circulation.

The epidemics of OHF in 1946–1947 and their decline during the following years can be explained by the situation that arose in Russia after the end of World War II. *D. reticulatus* and the narrow-skulled vole (*Microtus gregalis*) on which these ticks feed, inhabit open wild fields. After World War II, there was a shortage of manpower in Russia and large areas of arable land were left unfarmed, providing ideal conditions for *D. reticulatus* and the narrow-skulled vole to increase to very high levels. In subsequent years, the land was again cultivated, resulting in a progressive decrease of density of ticks and rodents and reduced incidence of OHF epidemics.

OHFV has also been isolated from mosquitoes, particularly *Aedes flavescens* and *Aedes subdiversus* that were trapped in regions where OHF outbreaks were registered among local hunters and muskrats during 1988–1991. Infected mosquitoes were only found in local regions surrounding the lakes where epidemics were occurring amongst the muskrats. It is worth noting that strains of OHFV isolated from ticks or mosquitoes differed in their neuroinvasiveness for laboratory mice, with lower indexes being recorded for mosquito-isolated strains. Mosquitoes are not considered important vectors of OHFV (Busygin, 2000).

As the result of these detailed epidemiologic and ecologic investigations, Russian scientists formulated the following explanation for the epidemiology of OHF (Busygin, 2000). OHFV circulates in areas where *D. reticulatus* and the narrow-skulled vole are present in significant numbers. These conditions occurred following World War II when fields were neglected. Muskrats were also involved in the process and became the source of virulent variants of OHFV for humans. Once the land was farmed, tick populations in these areas decreased and consequently the incidence of disease also decreased. It is now believed that the exclusive role of *D. reticulatus* and the narrow-skulled vole was only an episode in the epidemiology of OHF. However, outbreaks in muskrats still occur and serological surveys reveal human exposure to OHFV (Busygin, 2000). However, virus circulation is now primarily

found in areas surrounding lakes as opposed to the farmland-associated disease of the 1940s. As long as farmland continues to be cultivated, major tick-associated human epidemics are unlikely although there is still the potential for outbreaks associated with the fur trade, i.e., muskrats and nonvectored transmission. Recent data support this prognosis. During epidemic outbreaks of OHF during 1988–1997 in the Novosibirsk and Omsk regions, involving 165 cases, 10 of them were associated with ticks and 155 with muskrat hunters and poachers (Busygin, 2000).

B. Kyasanur Forest Disease Virus

Kyasanur Forest disease virus (KFDV) was first recognized in January 1957 at the Primary Health Centre, Ulvi, in the Shimoga District of Karnataka State in India. Reports of a large number of deaths with hemorrhagic symptoms, amongst monkeys, were investigated by virologists partly because humans gathering wood or grazing cattle and coming into contact with these monkeys also became fatally infected. Serologically, the virus was shown to be a member of the tick-borne encephalitis complex and this was subsequently confirmed by sequencing (Venugopal *et al.*, 1994). It is believed that the disease appeared after areas of the forest were cleared to extend the villages and grazing land for farm animals. The impact of deforestation and increased cattle grazing may have prolonged the time that indigenous monkeys spent on the forest floor, leading to increased exposure to KFDV-infected ticks in the undergrowth. The most abundant tick species in this region is *Haemaphysalis spinigera,* but the virus has also been isolated from seven other different species of *Haemaphysalis*, *Dermacentor*, and *Ixodes* ticks. In humans the virus produces severe hemorrhagic fevers with a 2–10% fatal outcome (Work and Trapido, 1957).

Although KFDV is genetically closely related to other mammalian tick-borne flaviviruses (implying an evolutionary connection with tick-borne flaviviruses found in Asia), until recently, KFDV appeared to be geographically isolated in the Karnataka State of India. However, the recent discovery and sequence determination (Charrel *et al.*, 2001) of a closely related subtype of KFDV, Alkhurma virus (ALKV), provides a rational explanation for the presence of KFDV in this single area of India. Alkhurma virus has been isolated several times since 1995, from the blood of patients with severe hemorrhagic fever in Saudi Arabia. Among 16 patients, four had lethal outcome. It therefore seems reasonable to propose that a group of closely related mammalian tick-borne viruses circulates in India and neighboring countries, at least as far

west as Saudi Arabia and even perhaps, in terms of evolutionary origins, into Africa. This proposal is supported by the presence of KADV (a more ancient lineage) in Africa and genetically closely related KSIV and RFV in Uzbekistan and Afghanistan. These observations imply that there may be many more subtypes of these tick-borne viruses in Africa, the Middle East, and central and southern Asia.

C. Reasons for Different Clinical Manifestations

Currently, there is no specific explanation for why some flaviviruses produce encephalitis while others produce hemorrhagic fevers. Despite the fact that sequences for these closely related encephalitic and hemorrhagic viruses are available (Charrel *et al.*, 2001; Gritsun *et al.*, 1993b; Venugopal *et al.*, 1994), it is not yet evident which part of the virus genome is responsible for these differences and what is the driving force for emerging viruses with these different pathogenicities for humans. A recent report noted that mosquito-borne viruses most frequently transmitted by *Aedes* spp. tend to cause hemorrhagic fevers in primate species whereas viruses most frequently associated with *Culex* spp. almost invariably cause encephalitis (Gaunt *et al.*, 2001). Analysis of the literature shows that all tick-transmitted flavivirus species, in particular, TBEV, LGTV, POWV, OHFV, and KFDV, were each isolated from different tick species although they may also share some vector species. Therefore one could speculate that amino acid differences between these viruses might be the result of adaptive evolution to particular tick species leading to the selection of different virus variants. This is also supported by the suggestion that the tick-borne viruses spend most of their time in ticks (Gould *et al.*, 2001), replicating only rarely in mammalian hosts. Nevertheless, sequence alignment of closely related "encephalitic" and "hemorrhagic" tick-borne and mosquito-borne flaviviruses has not yet revealed specific peptides that could be implicated in targeting to different organs and tissues in humans. On the other hand, pathogenicity of flaviviruses may depend upon a combination of specific amino acids within hypervariable domains of the envelope glycoprotein. It was proposed that changes in these amino acids may define the "pathogenic face" of flaviviruses (Gritsun *et al.*, 1995). Studies on dengue virus, a mosquito-borne flavivirus, revealed an alternative explanation. Antibodies against the NS1 non-structural protein of dengue virus cross-react with human fibrinogen, thrombocytes and endothelial cells, and the NS1 protein of DENV-2 binds to endothelial cells, implying a potential role of both "antigenic" and "biochemical" mimicry in dengue

hemorrhagic fever (Falconar, 1997). One could speculate that similar cross-reactivity between OHFV or KFDV-specific peptides with human vascular tissue proteins, rather than specific cell tropism, might induce hemorrhagic fever by damaging the blood vessels/capillaries. More data are required to understand the precise mechanisms of viral effects on pathogenesis of tick-associated flavivirus infections.

VI. Seabird Tick-Transmitted Viruses

Flaviviruses classified as the seabird tick-borne virus group were isolated from *Ixodes* and *Ornithodorus* spp. ticks that are commonly associated with seabirds. Three viruses are included in this group, namely Tyuleniy virus (TYUV), Saumarez Reef virus (SREV), and Meaban virus (MEAV) (Heinz *et al.*, 2000). These viruses were isolated from different geographical regions, TYUV from Russia, Norway, Alaska, and Oregon (Clifford *et al.*, 1971; Lvov *et al.*, 1971, 1972, 1975; Saikku *et al.*, 1980); SREV from Australia (St. George *et al.*, 1977); and MEAV from France (Chastel *et al.*, 1985). The unification of these viruses into one group and their separation from other flaviviruses was originally based on cross-reactivity in complement-fixation tests (Chastel *et al.*, 1985). Subsequent sequencing analysis demonstrated more close phylogenetic relationship between TYUV, SREV, and MEAV than with other viruses of the mammalian tick-borne group (Gould *et al.*, 2001; Marin *et al.*, 1995b), thus supporting the original antigenic relationships. From the estimated divergence times, they represent the most ancient lineage of the tick-borne flaviviruses. The genetic distance between each of these individual species is greater (approximately 60% amino acid identity) than that between most of the TBE complex viruses (approximately 70% amino acid identity), presumably reflecting greater biogeographic separation due to the migratory nature of seabirds (Gould *et al.*, 2001).

There are no reports of seabird or human diseases associated with these viruses, although serologic data show that the seabirds may be infected when they are bitten by infected ticks, indicating a long-term association between seabirds and the viruses (Gould *et al.*, 2001).

It has recently been suggested that Kadam virus (KADV), previously classified in the mammalian virus group (Heinz *et al.*, 2000), should be placed in the seabird virus group (Gould *et al.*, 2001), although it was isolated from ticks associated with mammals, namely from *Rhipicephalus gravus* feeding on cows in Uganda and *Hyalomma dromedarii* removed from a dead camel in Saudi Arabia. On the basis of sequence

data (Gaunt *et al.*, 2001; Kuno *et al.*, 1998), this virus is genetically more closely related to TYUV, SREV, and MEAV than to the TBE serocomplex viruses. Like the other seabird viruses, KADV probably does not cause disease and therefore does not play a significant role in flavivirus epidemiology.

VII. Control of Tick-Borne Encephalitis: Prospects for Live Attenuated Tick-Borne Encephalitis Virus Vaccines

Research into the epidemiology, biology, and ecology of TBEV provided the background information for the development of measures to control TBE. Although TBE appears no longer to be high on the list of priorities for control in Russia, before Perestroika it was considered a major problem and given high priority in the endemic parts of Russia. The following measures were used routinely in TBE endemic areas:

1. Broadcasting relevant information among the local people in endemic regions, concerning potential sources of TBEV infection, methods of avoiding tick-bites (wearing appropriate clothing, regular inspection for the presence of embedded ticks on the body), and measures to take if there was suspicion that a tick bite had occurred (reporting to medical authorities).
2. Owners of animals were reminded to treat their cats, dogs, etc., with acaricides. Local teams of men and women were used to cut the grass around houses before spraying the area with acaricides. Spraying extended to the nearby forested areas where local residents walked, camped, picnicked, etc.
3. Inactivated TBE vaccines were prepared and large numbers of individuals were immunized with these vaccines. The development of vaccine against TBEV in Russia has its own history, which will be described subsequently.

A. Inactivated Tick-Borne Encephalitis Virus Vaccines

The first attempts to develop a vaccine against TBE, in Russia, were made nearly 60 years ago using an inactivated vaccine prepared from TBE-infected mouse brains. The trials with this vaccine are reported to have reduced the incidence of TBE among vaccinated people by up to 10 times. Nevertheless the vaccine produced allergic reactions and failed to provide complete protection as shown by the fact that 6% of

vaccinees still became infected and developed TBE (Leonova, 1997; Zlobin and Gorin, 1996). Small-scale human trials of mouse-brain based vaccine with LIV were also reported in laboratory personnel in the United Kingdom. Specific antibody responses were detected in the vaccinees (Brotherston *et al.*, 1971; Edward and Takegami, 1993).

The next generation of inactivated vaccines that were prepared from tissue culture derived and purified virus did not produce allergic reactions, but were still ineffective in that the incidence of TBE cases in an endemic region was reduced by five times when 80% of the population was immunized (Leonova, 1997; Smorodintsev and Dubov, 1986; Zlobin and Gorin, 1996). Moreover, this vaccine failed to produce 100% protection against TBE in vaccinees that had received the full course of immunization. Medical surveys demonstrated longer incubation periods before the development of clinical symptoms among vaccinated people compared with non-vaccinated, but there was no difference in the severity of disease between these two groups. It was concluded that lack of complete protection was probably due to insufficient antigenic cross-reactivity between vaccine and endemic circulating TBEV strains.

Further development of inactivated vaccine was directed toward increasing the concentration of antigen to improve seroconversion and reduce the amount of non-viral proteins that produce allergic reactions. These concentrated and purified vaccines were first developed in Austria (Kunz, to be published; Kunz *et al.*, 1976, 1980) and then in Russia (Chumakov *et al.*, 1991). The Austrian vaccine is now commercially available from two producers in Austria and Germany (Barrett *et al.*, 1999).

In Austria, the vaccine was used extensively and although no controlled clinical trials were reported, it is considered to have performed efficiently on the basis of the high levels of seroconversion and the progressive reduction of numbers of cases of TBE. Moreover, it has been shown to be extremely safe for use in both adults and children. The vaccine established long-lasting immunologic memory because specific antibodies were detected in a high proportion of people 3–6 years after the last vaccination (Barrett *et al.*, 1999). Only a small fraction of vaccinees developed minor side effects, justifying the high level of vaccine purification that has been applied to this vaccine. A pediatric variant of the vaccine containing a smaller dose of antigen was also developed and is recommended for very young children (from 6 months in high risk regions) (Kunz, to be published). Since 1980, 35 million doses of vaccine have been used, 6.8 million people have been vaccinated in Austria, and the estimated rate of protection is 96–99% in vaccinees

who have completed the immunization protocol of three doses. All rare recorded incidences of TBE among vaccinees have either been related to cases with an incomplete course of vaccination or have occurred in people older than 60 years of age (Barrett *et al.*, 1999; Kunz, to be published). The increasing vaccination coverage resulted in a steady decline of the morbidity of TBE in Austria, and in the previous 3 years 41, 60, and 54 cases of TBE were recorded annually in contrast with the neighboring Czech Republic and Slovakia, where intensive vaccination programs have not been applied. In these cases, over 700 TBE cases have been registered annually for the last several years (Dr. M. Labuda, personal communication). New formulations of the Austrian vaccine without stabilizers such as human albumin and preservatives such as thimerosal have been developed to reduce the low level of side effects even further. The removal of thimerosal from the vaccine had been requested by WHO in relation to possible vaccine-associated autism in children (www.autism-mercury.com/). Moreover, thimerosal contains 49.6% mercury by weight and possibly therefore could act as a neurotoxin. Nevertheless, the removal of human albumin as a stabilizer from the vaccine resulted in increased adverse effects in children, particularly febrile convulsions, probably due to the elevated synthesis of cytokines (Marth and Kleinhappl, 2001) and currently human albumin has been re-introduced into the TBEV vaccine.

Overall, the Austrian experience has demonstrated that following intensive immunization programs and a systematic approach, the disease can be almost eliminated (Kunz, to be published).

In Russia, a similar vaccine had been produced and used in small-scale human trials (600,000 people). Laboratory experiments with mice showed the same level of protection as the Austrian vaccines (Vorob'eva *et al.*, 1996). In the human trials, the vaccine was found to be highly immunogenic and to have low reactogenicity (Chumakov *et al.*, 1991). Nevertheless because of the high cost, the application of the concentrated purified Russian vaccine has been limited mainly to laboratory personnel, whereas a non-concentrated tissue culture vaccine based on another TBE strain, 205, is still in use in many Russian regions and outperforms the earlier vaccine based on TBEV strain, Pan. Although the vaccine in Austria has been shown to be very effective, it does not necessarily follow that a similar performance would be achieved in Russia, which presents very different problems. First, many of the villages, towns and cities are located among the forests and the risk of exposure to ticks and TBE is consequently very high. Second, TBEV strains circulating in Russia belong to the Siberian and Far Eastern subtypes. They are more virulent than strains of

the European subtype that is endemic in Western Europe. Tick-borne encephalitis is therefore still a major problem in Russia requiring significant medical resources and research efforts. Even in the period before "Perestroika," when intensive anti-TBE measures, including mass-vaccination, were in force, there were still 700–1200 registered TBE cases annually.

There are several possible explanations for the failure of Russian vaccines to afford high levels of protection. First, strain heterogeneity probably provides an opportunity for viruses to escape the immunity established following vaccination (Leonova, 1997). So-called "antigenically defective" strains of TBEV have been isolated from ticks and patients in endemic regions of European Russia that react poorly with commercially available diagnostic kits (Pogodina *et al.*, 1992). Because no sequence information has been produced for these viruses, it is difficult to conclude how many TBEV strains are circulating in Russia. One possible solution to this problem would be to develop a polyvalent vaccine by using a mixture of different strains of TBEV. However, this would require significant resources and a highly developed medical infrastructure.

One of the major problems associated with inactivated TBEV vaccines in Russia is the need to administer them at least twice within the first year and then at least once every 3 subsequent years. This raises problems of logistics and cost. Another problem associated with inactivated vaccines relates to the use of formaldehyde, which in theory may destroy epitopes responsible for inducing protective immune responses. In this respect the use of recombinant technology for the production of subviral particles and nonstructural proteins that does not require inactivation of virus and therefore preserve better antigenic structure of immunogens might overcome this problem in the future (Dmitriev *et al.*, 1996; Heinz *et al.*, 1995; Holzer *et al.*, 1999).

A third possible problem with inactivated vaccines is allergic reactions developing after several booster doses have been administered. Improvement of purification protocols in Austria reduced the side effects and led to the production of a vaccine that is safe for very young children (Barrett *et al.*, 1999). New types of vaccines using naked DNA (Aberle *et al.*, 1999; Schmaljohn *et al.*, 1999) and synthetic infectious RNA (Mandl *et al.*, 1998a) are under development. Because these vaccines are produced *in vitro*, they contain no host proteins and therefore do not induce allergic reactions and require less complicated purification protocols.

B. Trials of Live Attenuated Langat Virus–Based Vaccines

To overcome the problems associated with inactivated virus vaccines, several attempts were made to produce a live-attenuated vaccine in Russia, based either on naturally attenuated (serial subculture) or mutated TBEV and LGTV that were tested in primates and in some cases on human volunteers (Pogodina *et al.*, 1986; Smorodintsev and Dubov, 1986). None of the vaccines satisfied standard safety requirements despite the fact that they all failed to produce clinical symptoms in monkeys following intracerebral inoculation. In every case, subsequent pathomorphologic analysis showed evidence of persistent/chronic infection in the different organs and tissues (Pogodina *et al.*, 1986).

The most well known clinical trials used LGTV strain TP21, which was considered to be the most attenuated virus for mice and primates. Several attempts were made to isolate reliably attenuated variants of TP21 and one of them, Elantcev 15-20/3, was used to vaccinate 649,470 Russian volunteers. Thirty-five vaccinees developed meningoencephalitis resulting in permanent neurologic sequelae (Leonova, 1997; Smorodintsev and Dubov, 1986; Zlobin and Gorin, 1996). Thirty-two of these cases arose following vaccination of 379,540 individuals with live vaccine without a preliminary injection of inactivated vaccine. However, in another group of 269,939 individuals who were given inactivated TBEV vaccine before the administration of live-attenuated vaccine, encephalitis developed in three people. Langat virus was also used to treat cancer patients; encephalitis developed in two of 36 vaccinated individuals (Smorodintsev and Dubov, 1986). As a postscript to this report, it is worth noting that although laboratory experiments with the candidate vaccine virus Elantcev 15-20/3 did not produce death in any of the monkeys, following intracerebral inoculation, 30% of the animals developed neurologic lesions from which they subsequently recovered (Smorodintsev and Dubov, 1986).

Retrospective analysis was carried out to establish the efficacy of these trials in more than 30,000 of the humans vaccinated with Elantcev 15-20/3 in the Perm and Sverdlovsk regions of Russia. The individuals were monitored 20 years after the vaccination program described above (Shapoval *et al.*, 1989). The study showed that live vaccine reduced the incidence of TBE in endemic regions by more than 10 times in comparison with an equivalent group of individuals given inactivated vaccine, and by more than 20 times in comparison with a control group given a placebo. A single immunization with live vaccine produced seroconversion in 100% of individuals. Inactivated vaccine in

Russia was less efficient, requiring multiple vaccinations to achieve a 90–100% immune response. In addition, 100% and 74% of vaccinees were still serologically positive 2 and 3 years, respectively, after immunization with live-attenuated vaccine. On the other hand, in Russia only 25–40% were positive 6 months after the last revaccination with inactivated virus.

Besides Elantcev 15-20/3, other LGTV-based clones were used in a separate series of small-scale human trials with approximately 3000 volunteers and none of them produced harmful side effects but it is very difficult to draw conclusions with these limited data. Before the large-scale trials described above, an experiment with Elantcev 15-20/3 was performed on 28,000 volunteers with no side effects or complications. Pathomorphologic assays in monkeys, carried out before the human trials with Elantcev 15-20/3 and other clones, did not reveal significant differences between any of these strains in neuronal lesions.

A retrospective analysis that took place approximately 20 years post-vaccination focused on somatic disease amongst 1186 people in the Perm region and 3710 people in the Ekaterinburg region. The control group included people who had never been vaccinated with LGTV vaccine. The results showed that there has been no increase in the incidence of the major human diseases (heart attacks, strokes, cancer, infections, etc.) in the vaccinated group compared with control non-vaccinated individuals. An earlier survey of 7223 vaccinees did not reveal an increase of general infectious diseases (flu, cold, pneumonia, fevers) in the first year after the vaccination in comparison with the control group. In fact the lowest level of infectious diseases was registered in the group vaccinated with LGTV.

In spite of these advantages, human trials with live attenuated vaccines based on LGTV have also revealed three major problems. One problem is the failure to provide 100% protection against TBE infections in endemic regions. An explanation could be that defects in the immune system, encountered in humans for a variety of reasons, including stress, alcoholism, pregnancy, etc., lead to poor seroconversion. An alternative explanation is the absence of appropriate antigenic cross-reactivity between LGTV and local TBEV strains that in conjunction with reduced levels of immunity, fail to provide appropriate protection. Langat virus, which is a different virus species from TBEV, shares only 82–88% of amino acid identity with TBEV, whereas within the TBEV group the lowest identity is 95–96% (Gritsun *et al.*, 1993a, 1993b).

The second problem of using the LGTV-based vaccine is the high incidence of encephalitis of 1:10,000, indicating that the strain chosen for the clinical trials was not suitable. Subsequently a pathomorphologic assay that enabled differentiation of various low-pathogenic strains of LGTV following spinal inoculation of monkeys was developed. This assay demonstrated higher neurovirulence of Elantcev 15-20/3 in comparison with the original LGTV TP21 strain or other clones selected from the original TP21 population (Kamalov *et al.*, 1993; Sokolova *et al.*, 1994).

A third problem arises from the potential capacity of LGTV to establish persistent infection in cell culture and in hamsters (Boulton *et al.*, 1971; Illavia and Webb, 1969; Zlotnik and Grant, 1975; Zlotnik *et al.*, 1973). The development of chronic disease in a few human cases following vaccination with LGTV has also been reported (Pogodina *et al.*, 1986). Nevertheless, a retrospective analysis on 15,318 individuals about 20 years later demonstrated the absence of any chronic neurologic disease (Shapoval *et al.*, 1989), and this is why a LGTV-based vaccine is still being considered in Russia for use in the future. However, the potential for other live virus vaccine candidates to cause persistent infections must be taken into account and investigated thoroughly (Pogodina *et al.*, 1986).

Another potential problem for live-attenuated vaccine in Russia arises from reports of seronegative cases of TBE, which were diagnosed on clinical grounds only (Leonova, 1997; Pogodina *et al.*, 1992). It was suggested that circulating antigenically defective TBEV strains that fail to react with commercially available diagnostic reagents (Pogodina *et al.*, 1992) could be responsible for this phenomenon (see also Section VII,A). Nevertheless, in some robustly documented cases antigenically authentic TBEV strains have been isolated from patients that did not develop antibodies during the virus infection. Additionally, a proportion of people in endemic regions have not shown any immunologic response following vaccination with inactivated or LGTV-based live vaccine (Prof. V. A. Lashkevich, personal communication). For example, the Vasilchenko TBEV strain (Siberian subtype) was isolated from the blood of a patient with a subacute case of TBE from which he completely recovered. Antibodies have never been revealed during or after the disease following 11 years of observation, although during this period he was also vaccinated with live LGTV-based vaccine (Pogodina *et al.*, 1986).

Another possible explanation for seronegative cases of TBE in endemic regions could have an immunologic basis. Immunological

tolerance may develop in adults following exposure of neonates to antigen, due to irreversible inactivation of appropriate T-cell clones (Borenstein *et al.*, 2001; Janeway *et al.*, 1996). Immunologic tolerance acquired neonatally in experimental conditions has been demonstrated following virus infection (Cihak and Lehmann Grube, 1978; Kobayashi, 1975; Kobets *et al.*, 1995; Mauracher *et al.*, 1993; Seto *et al.*, 1988; Watts *et al.*, 1999; Wohlsein *et al.*, 1992). As described previously, more than 70–95% of TBEV human infections are subclinical and therefore one could speculate that if a mother became infected with TBEV during pregnancy, then her child could develop immunological nonresponsiveness to the virus. Another reason for seronegative TBEV cases could be immunological suppression following interactions between viruses and cells of the immune system such as dendritic cells (Fugier Vivier *et al.*, 1997; Oldstone, 1998; Sevilla *et al.*, 2000). The absence of an antibody response in some patients might be explained by variation in the permissiveness of immunocompetent cells of different individuals to virus. Perhaps, for example, immune cells of seronegative individuals support cytolytic virus infection.

Thus a proportion of people in endemic regions may not be protected even against low virulence TBEV strains and therefore administration of live-attenuated vaccine could be a dangerous strategy. All these facts have to be weighed against each other when live attenuated vaccines are being developed in the future.

C. New Advances Toward Live Attenuated Tick-Borne Encephalitis Virus Vaccines

Previous trials of live attenuated vaccines based on LGTV in the early 1970s demonstrated that the vaccine strain failed safety requirements because it was not attenuated sufficiently. New developments in molecular virology during the 1990s, especially the availability of sequencing techniques and infectious clones, have begun to explain many aspects of virus attenuation and offer methods to engineer custom-designed viruses with predictable pathogenicity. Such developments have opened up new approaches for producing safe and effective live virus vaccines. These are listed below.

- The virus should not establish persistent infections or produce chronic disease.
- The virus must be reliably attenuated to reduce the possibility of reversion to virulence during replication in the host.

- A balance has to be achieved between attenuation and induction of immune response, for appropriate protection.
- The virus should not target nerve cells.

The molecular basis of chronic disease and TBEV persistence has not been determined. Hence we still do not know which parts of the TBEV genome are responsible for the difference between persistent and non-persistent strains. This question is closely related to the problem of flavivirus attenuation and ultimately to the production of live virus vaccine. Attenuation and persistence probably share the same genetic determinants and molecular mechanisms that are involved in virus reproduction. The most well-known mechanism of virus attenuation is alteration of different virus functions through single point mutations in any gene or untranslated region. This has been demonstrated for many flaviviruses (Allison *et al.*, 2001; Amberg and Rice, 1999; Cahour *et al.*, 1995; Cecilia and Gould, 1991; Gritsun *et al.*, 1990, 2001; Mandl *et al.*, 1998b; Proutski *et al.*, 1997; Xie *et al.*, 1998). However, the live-attenuated vaccines for two mosquito-borne flaviviruses, YFV and JEV, both of which were derived empirically, have multiple substitutions over the entire virus genome in comparison with their wild-type parent viruses (Aihara *et al.*, 1991; dos Santos *et al.*, 1995; Hahn *et al.*, 1987; Rice *et al.*, 1985; Wills *et al.*, 1993; Xin *et al.*, 1988). This supports the widely held view that reliable attenuation of vaccine viruses is achieved through the cumulative effect of many point mutations. This principle has been demonstrated for TBEV. Five point-mutations that mapped in different regions of the genome, each produced a small biologic change, that was not always detectable in all biologic reactions, but the accumulative effect of these mutations led to attenuation of the virus (Gritsun *et al.*, 2001). Equivalent findings were also reported for the chimeric YF/JE virus (Arroyo *et al.*, 2001). The theoretical advantage of attenuating a virus using a series of point mutations, each of which contributes only a small amount to attenuation, would be to reduce the risk of reversion mutations that exert a strong virulence effect.

As a result of multiple point mutations, all existing live vaccine viruses show reduced capacity to produce high levels of infectious virus in their hosts. This is a major factor that determines attenuation and protective activity of vaccine viruses. Nevertheless, attempts to attenuate TBEV frequently led to the production of viruses with the ability to establish persistent infections *in vivo* and, in practice, it has proved difficult to achieve a balance between attenuation and the absence of virus persistence (Pogodina *et al.*, 1986). Moreover, the increase in

virus attenuation frequently results in a reduction in protective activity as a result of weaker antigenicity or impaired virus production.

Several TBEV infectious clones have been constructed to study the different aspects of TBEV pathogenesis and to develop live-attenuated vaccines (Gritsun and Gould, 1998; Mandl *et al.*, 1997; Pletnev, 2001). Two of them represent European subtype strains of TBEV (Mandl *et al.*, 1997). The other was produced from Vasilchenko virus, an endemic virus strain circulating in Europe and Siberia (Gritsun and Gould, 1998; Gritsun *et al.*, 2001; Hayasaka *et al.*, 2001; Lundkvist *et al.*, 2001; Mavtchoutko *et al.*, 2000). The Langat virus and its more attenuated variant E5 were also used to produce infections clones (Pletnev, 2001). Each of these infectious clones can be used as a basis for further virus attenuation by introducing multiple point mutations, each with a small change in virus phenotype. As pointed out previously, this strategy reduces significantly the risk that the virus will revert to virulence.

Although the molecular mechanisms of TBEV persistence have not yet been identified, apoptosis, an active process of programmed cell death (Griffin and Hardwick, 1999; Griffin *et al.*, 1994; Shen and Shenk, 1995), might have a significant role in this process. For example, the establishment of alphavirus persistence was associated with the balance between vertebrate proteins bcl-2 and the recognized cellular mediators of most apoptotic pathways (Griffin and Hardwick, 1997). To date, more than a dozen virus genomes have been shown to encode activities that modulate apoptosis. Some virus-encoded products prevent apoptosis whereas others trigger apoptotic cell death thereby contributing to the pathogenesis of virus infections. The ability to suppress apoptosis enables the virus either to establish persistent infection or prolong the time available for lytic replication to maximize the yield of virus progeny (Hashimoto, 1996), which could be the case for persisting TBEV.

The relevance of apoptosis to acute TBEV and LGTV infections has been reported (Isaeva *et al.*, 1998; Kamalov *et al.*, 1998; Prikhod'ko *et al.*, 2001). Although the virion envelope protein of LGTV has been demonstrated to induce apoptosis (Prikhod'ko *et al.*, 2001), the precise epitopes that provide this activity have not yet been identified. Other viral proteins could also be involved in the apoptotic activity of TBEV. It is important to note that the tick, which is the source of virus in the natural environment, also supports TBEV replication for months or years without obvious damage to any tissues. Perhaps tick survival results from the presence of anti-apoptotic determinants in TBEV proteins that suppress programmed cell death in a cell-specific manner.

A search for such types of activity in mammalian cells might lead to a strategy for vaccine development in which specific pro- or anti-apoptotic determinants are incorporated into an infectious clone constructed from a candidate vaccine virus. The objective would be to reduce the capacity of TBEV to establish persistence in the host and limit virus production in infected cells.

Understanding the molecular basis of attenuation and persistence might also resolve the problem of TBEV neurovirulence. The most common feature of many flaviviruses is their ability to target and replicate in viscerotropic organs of wild animals without damaging them. Virus invasion and damage of the CNS does not provide an obvious evolutionary advantage for the transmission triangle (tick-virus-vertebrate), and as a rule wild vertebrate hosts of ticks in endemic regions are resistant to TBEV infection. Virus-induced damage to the CNS occurs inadvertently in humans and some animals that are not part of the evolutionarily stable host-pathogen communities, and their survival is not important for virus transmission. Nevertheless the question of whether or not TBEV in nature invades the CNS of resistant animals is highly intriguing and has not been investigated thoroughly. Infectious TBEV has been found in the brain of healthy local rodents and experimental mice (Kozuch *et al.*, 1995; Shapoval, 1976; Vargin and Semenov, 1985). In Syrian hamsters the virus has been isolated from the brain of healthy animals (Frolova *et al.*, 1982a, 1987), supporting the view that these viruses have probably acquired neuron-specific anti-apoptotic determinants that reduce virus neurovirulence.

As argued previously, the pathogenetic characteristics of European, Siberian, and Far Eastern TBE subtypes appear to differ. Clinical manifestations of TBEV and experimental modeling of encephalitis in animals demonstrate a higher degree of neurovirulence for Far Eastern TBEV in comparison with the European subtype. It was suggested that the occasional occurrence of damage to the CNS produced by European strains of TBEV results from secondary inflammatory processes induced by the virus in mesenchymal tissues of the brain, rather than from direct virus neurotropism. In contrast, Far Eastern TBEV damages the CNS because it directly targets motor neurones as sites of primary virus reproduction (Votiakov *et al.*, 1978). It is therefore most likely that virus attachment or entry to motor neurons determines the different neurovirulence of these viruses. The differences between European and Far Eastern TBEV in sensitivity to sulphated polysaccharides (Dzhivanian and Lashkevich, 1970) also support this view. On the other hand, the presence of viruses of the Siberian

subtype in the motor neurons of healthy hamsters surviving for 2 years after virus challenge (Frolova *et al.*, 1982a, 1987) suggests that Siberian virus Vasilchenko has a genetically predetermined ability to suppress neuronal apoptosis. Identification of the mutations in TBEV that eliminate the capacity to target neuronal cells and to produce severe apoptosis would be a major step forward in the development of safe and effective live-attenuated vaccines.

The first large human trials with live-attenuated vaccine based on LGTV were unsuccessful. Although LGTV is not the best choice as a future vaccine candidate because of its distant serologic and phylogenetic relation to TBEV, retrospective analysis among vaccinees revealed several advantages for the use of live-attenuated vaccines in comparison with inactivated virus. These include superior seroconversion and more effective protection. A deeper understanding of the molecular basis of TBEV attenuation, persistence and neurovirulence, will enable scientists to design and construct viruses that satisfy the most stringent criteria for safety and reliability of future attenuated vaccines.

VIII. Conclusions

This chapter highlights the major epidemiologic features of tick-borne encephalitic virus infections, focusing particularly on Russia where most cases (about 11,000) of human infections are recorded each year. The increase in TBE incidence is largely associated with deterioration of the economic situation in Russia after "Perestroika," but even in the years when pesticide control and vaccination programs were in operation, there were still approximately 700–1200 cases of TBE observed annually. It is still not understood why TBE cases are encountered among vaccinated people, and this chapter discusses the possible factors that influence incidence and severity of the disease. These unresolved problems highlight the necessity for further research into the pathogenicity, epidemiology, and molecular properties of tick-borne flaviviruses before we can effectively develop rational control methods.

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ADVANCES IN VIRUS RESEARCH, VOL 61

EPIDEMIOLOGY OF OTHER ARTHROPOD-BORNE FLAVIVIRUSES INFECTING HUMANS

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I. Introduction

Many of the chapters in this book deal with flaviviruses that cause major diseases in humans and animals. These viruses include dengue fever, St. Louis encephalitis, West Nile encephalitis, Japanese encephalitis, Murray Valley encephalitis, tick-borne encephalitis, and yellow

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fever virus. This chapter describes 20 other flaviviruses infecting humans, each of which is characterized either by focal geographical distribution, or by very low clinical attack rates. These viruses and the diseases they cause may be of intense local interest. However, they do not generate research support outside of their own region or attract the attention of vaccine developers because the commercial market is not lucrative. On the other hand, they have the potential to emerge as important threats should viral mutation or ecological change take place. The world was reminded by the appearance of West Nile virus in New York City in 1999 that flaviviruses also have the potential to be translocated to more favorable geographical sites and emerge in epidemic form. For these reasons, these other flaviviruses merit discussion and attention.

Table I outlines the geographical area, vector, and syndromes associated with human infections by 20 other flaviviruses. It is instructive to examine these viruses with respect to the continent where they are encountered. Geographically localized flaviviruses of Asia and North America are tick-borne. Flaviviruses of Africa, South America, and Australia/New Guinea are mosquito-borne. Syndromes associated with these virus are variable. Most of these viruses have been recognized only in febrile patients; some patients also have rash or arthritis. The tick-borne viruses cause encephalitis, and two (Kyasanur Forest disease and Omsk hemorrhagic fever) also are associated with hemorrhagic fever.

II. Other Flaviviruses of Africa

A. *Banzi Virus*

Banzi virus was isolated from the blood of a febrile child in Tongaland, South Africa (Smithburn *et al.*, 1959). The agent was also recovered at about the same time from a pool of *Culex rubinotus* mosquitoes sampled near Johannesburg. These are the only reported isolates of Banzi virus. The virus is closely related serologically to Uganda S virus, which is not known to cause human disease.

Banzi virus infection of genetically susceptible and resistant C3H mice has been used as a model to study the genetic basis for resistance to development of encephalitis (Jacoby *et al.*, 1980). Protection was dependent on thymic-derived lymphocytes. Tissues from resistant and susceptible mice were equally susceptible to infection with Banzi virus *in vitro*.

TABLE I
"OTHER" FLAVIVIRUSES ASSOCIATED WITH HUMAN DISEASES

Continent	Virus	Vector	Syndrome
Africa	Banzi	mosquito	fever
	Koutango	mosquito	fever, rash
	Spondweni	mosquito	fever
	Usutu (also found in Europe)	mosquito	fever, rash
	Wesselsbron (also found in Asia)	mosquito	fever
	Zika (also found in Asia)	mosquito	fever
Europe	Louping ill	tick	encephalitis
Asia	Karshi	tick	fever, encephalitis
	Kyasanur Forest disease	tick	fever, hemorrhage, meningoencephalitis
	Omsk hemorrhagic fever	tick	hemorrhage, encephalitis
	Negishi	unknown, probably tick	encephalitis
N. America and Asia	Powassan	tick	encephalitis
N. America	Rio Bravo	unknown	fever
	Modoc	unknown	fever, aseptic meningitis
S. America	Bussuquara	mosquito	fever
	Ilheus	mosquito	fever, encephalitis
	Rocio	mosquito	encephalitis
Australia	Edge Hill	mosquito	fever, arthritis
	Kokobera	mosquito	fever, arthritis
	Sepik (found only in New Guinea)	mosquito	fever

B. Koutango Virus

Koutango virus was recovered from an African rodent, *Tatera kempi*, trapped in 1968 in Koutango village, Senegal. This area is sparsely forested and supports cultivation of peanuts and millet. Complement fixation tests showed a relationship with West Nile virus. Koutango virus has since been found in *Mastomys* sp. and *Lemnyscomys* sp. in Senegal and the Central African Republic (Karabatsos, 1985). A Russian team also reported finding the virus in Somalia (Butenko,

1986). A single human infection occurred in a laboratory worker in Senegal. The patient had fever and a rash. No isolates have been made from arthropods; however, the virus was transmitted by *Aedes aegypti* from mouse to mouse, and Koutango virus was also transmitted to mice by mosquitoes infected transovarially.

C. Spondweni Virus

Spondweni virus was isolated from a febrile human being in Nigeria during an outbreak of disease with jaundice (Macnamara, 1954). Previously, Spondweni virus had been recovered from pools of mosquitoes in Tongaland, including *Ae. circumluteolus, Cx. univittatus, Mansonia africana, Ae. cumminsi*, and *Eretmapodites silvestris*, as well as from two febrile laboratory workers (McIntosh *et al.*, 1961) and from *Ma. uniformis* in Natal in May of 1955 (Kokernot *et al.*, 1957). Single human cases were recorded in Cameroon and Upper Volta. The disease is characterized by fever, chills, headache, malaise, nausea, and epistaxis and in one case a rash (Karabatsos, 1985; Wolfe *et al.*, 1982). The reservoir is not known, although in the laboratory wild African rodents developed viremia, and the virus was transmitted to mice by *Ae. circumluteolus* mosquitoes.

D. Usutu Virus

Usutu virus is serologically closely related to Japanese encephalitis and West Nile viruses. It was first recognized in Natal, South Africa, in 1959, isolated from *Cx. neavei* in the tropical coastal lowland. The virus was isolated only once from a patient in the Central African Republic, who had fever and rash. It was recovered on multiple occasions from mosquitoes, mostly *Culex* spp., and from passerine birds. It was also recovered once from a rodent, *Praomys* sp. Usutu virus is widely distributed in Africa and has been isolated from mosquitoes or birds in Uganda, Cameroon, Senegal, and Nigeria (Karabatsos, 1985).

During the summer of 2001 in Austria, birds were found dead and dying. A virus was isolated from the brains of the birds and was identified by reverse transcriptase polymerase chain reaction as Usutu virus. The nucleic acid similarity was 97%. No human disease was recognized. The finding strengthens the case for birds as part of the reservoir for Usutu virus and also favors the idea that there may be intercontinental movement of the virus (Weissenbock, 2002).

E. Wesselsbron Virus

Wesselsbron virus causes an acute mosquito-borne infection of sheep, goats, and cattle. It also uncommonly infects humans. The infection in sheep was first recognized in 1955 in Orange Free State, South Africa, when neonatal deaths and abortions, thought initially to be an adverse reaction to vaccination for Rift Valley fever, yielded Wesselsbron virus on culture. Later that year, a field worker with a medical team in Natal developed fever and viremia due to Wesselsbron virus. Mosquitoes in Natal also were shown to be infected. Additional isolates of the virus were made in 1957 from a lamb in the Kroonstad district; in 1956 and 1957 from sheep, mosquitoes, and an entomologist in Middleburg of the Cape Province; in 1973, 1974, and 1975 from cattle in Natal and Transvaal; in 1975 from lambs in the Orange Free State; and in 1988 in lambs from Cape Province and the Orange Free State (Swanepoel and Coetzer, 1994).

Outside of South Africa, the virus was recovered in Zimbabwe from mosquitoes in 1969–1970 and from a cow in 1978. A study in Madagascar in 1989 demonstrated the infection of *Ae. circumluteolus* mosquitoes with Wesselsbron virus. These mosquitoes were sampled in primary forest, and the finding supports the hypothesis that there is a forest cycle of the virus infection (Morvan *et al*., 1990). Infection was also documented in West Africa in mosquitoes, a camel, and humans and in Thailand from mosquitoes (Swanepoel and Coetzer, 1994).

The disease in humans is not considered to be life-threatening, although the patients are acutely ill for about 3 days, followed by at least a week of convalescence, during which there is loss of appetite, malaise, and lassitude. The usual course of illness after 2 to 7 days of incubation is sudden fever, headache, rapid heart rate, myalgia, and arthralgia. A minority of patients have had loss of visual acuity, rash on the trunk, cutaneous hyperesthesia, enlarged and tender liver, and a rise in hepatic enzymes. An unusual human case followed an accident in the laboratory in which the subject was splashed in the eye by a virus-containing fluid. Symptoms and signs indicated a probable encephalitis with severe headache, speech abnormality, deafness, muscle spasms, and incoordination (Swanepoel and Coetzer, 1994).

The disease in livestock is most prominent in cattle, sheep, and goats. Abortion and death of newborn animals is common in sheep and goats. Congenital malformations including arthrogryposis are seen in newborn sheep and cattle. Adult animals usually undergo inapparent infections. A partially attenuated live vaccine is used in South Africa and is effective when administered before the breeding season. The

vaccine causes *hydrops amnii*, however, especially if given to ewes in the first trimester of pregnancy, and is associated with arthrogryposis and anencephaly.

Multiple isolations of Wesselsbron virus from *Aedes* and *Ochlerotatus* mosquitoes implicate these strongly as vectors during outbreaks of disease in domestic animals. In South Africa, *Ae. mcintoshi* and *Ae. luridus* and *Oc. Juppi* and *Oc. caballus* are epizootic vectors. The vertebrate reservoir host is not determined. Viremia occurs in birds experimentally infected in the laboratory (Woodall, 2001).

F. Zika Virus

Zika virus was recovered from blood of a sentinel rhesus monkey exposed in 1947 in the canopy of Zika Forest, near Entebbe, Uganda (Dick *et al.*, 1952). Human cases are rarely reported, although antibody studies provide evidence that human infection is widespread throughout Africa and Asia.

The human disease is characterized by fever, headache, malaise, and a maculopapular rash. There have been only five documented cases: three naturally acquired in Nigeria and one each in field and laboratory workers in Senegal and Uganda.

Zika virus has been isolated repeatedly from mosquitoes. The virus was recovered from *Ae. africanus* in Uganda and the Central African Republic, *Ae. luteocephalus* in Nigeria, *Aedes* sp. in Cote d'Ivoire and Senegal, and *Ae. aegypti* in Malaysia. Some of the isolates in Uganda were from mosquitoes attracted to humans in the forest canopy (Karabatsos, 1985). It is thus possible that there is both a forest canopy cycle of transmission, as well as a terrestrial cycle.

III. Other Flaviviruses of Europe

A. Louping Ill Virus

Louping ("leaping" in Scottish dialect) ill virus was isolated from pooled brains and spinal cords of a lamb and ewe in 1929 on a tick-infested hill farm in Selkirkshire, Scotland. The animals had incoordinated gait, champing of the jaws, salivation, and later prostration and vigorous kicking. Louping ill has been known as a disease of sheep in Scotland for two centuries. It also occurs in the rest of the United Kingdom, Ireland, and Norway and perhaps other European countries (Karabatsos, 1985; Swanepoel, 1994).

The disease is primarily one of sheep; however, other domestic animals may be infected and manifest only mild disease. The virus also infects persons occupationally exposed to the bite of a tick. Disease in humans is rare. Five laboratory acquired and eight naturally infected cases were reported, and it is estimated that there have been about 40 in total. The disease is biphasic, characterized by fever, headache, photophobia, altered sensorium, nausea, rash, and muscle and joint pain. During the second phase of the illness, patients have severe headache, confusion, delirium, and drowsiness, sometimes leading to coma. White blood cells are found in the cerebrospinal fluid. There have been no fatalities, but convalescence may take up to 3 months. An inactivated vaccine is available for sheep, but there is no vaccine for human use.

The virus is maintained in nature in *Ixodes ricinus* ticks, which are abundant in the unimproved pastures. *I. rincus* ticks feed on a wide variety of vertebrate animals. *I. ricinus* has two populations, one feeding on vertebrate animals only in the spring and the other feeding only in the fall. Consequently, there are two peaks of disease in sheep. Red grouse, *Lagopus lagopus scoticus*, are susceptible to infection and disease. Small mammals such as *Apodemus sylvaticus* and *Sorex araneus* may play a role in virus maintenance. The Blue Mountain hare, *Lepus timidus scoticus*, is susceptible to tick co-feeding transmission without viremia. This form of transmission occurs when an infected tick feeding on the same animal as a noninfected tick transmits louping ill virus in the absence of viremia in the vertebrate host (Gould, 2001).

IV. Other Flaviviruses of Asia

A. *Karshi Virus*

Karshi virus was first isolated in Uzbekistan from *Ornithodoros papillipes* in 1972. These ticks were in the burrows of *Rhombomys opimus*, the Great Gerbil. The landscape is dry subtropical. The virus was subsequently isolated in Kazakhstan from *Hyalomma asiaticus* and *Dermacentor marginatum* ticks and from the rooks, *Corvus frugilegus* and *R. opimus* (Lvov, 1993).

Human infection is sporadic. Seven laboratory confirmed cases were then investigated. These were characterized by fever reaching 39.8 °C and tapering off over a period of 1 week.

The reservoir for Karshi virus is not established. Laboratory inoculation of arthropods favor tick transmission. The virus persisted at least 60 days in inoculated *H. asiaticus* and was found in the ovaries

and salivary glands. The virus also replicated in mosquitoes but to a lower titer that in ticks. It is thus possible that ticks are the reservoir. It is unusual for a flavivirus to replicate in both ticks and mosquitoes, but West Nile virus, a close relative of Karshi virus, also replicates in both sets of arthropods.

B. Kyasanur Forest Disease

An outbreak of human hemorrhagic fever first came to the attention of authorities in March 1957 in the Kyasanur Forest region of southwestern India. The association of human deaths with mortality in monkeys suggested that yellow fever had somehow been introduced, which would have been an international emergency. The Indian authorities, with assistance from The Rockefeller Foundation, reacted swiftly at the Virus Research Centre in Pune to isolate a flavivirus named Kyasanur Forest disease (KFD) virus, new to science and belonging to the tick-borne encephalitis complex.

The incubation period in humans is 3 to 8 days after the bite of an infected tick. Sudden onset of fever up to 40 °C is accompanied by muscle aches, headache, cough, diarrhea, malaise, and conjunctivitis. An enanthem consisting of papules and vesicles on the soft palate is characteristic. Hemorrhagic manifestations were reported in early studies, but more recently, hemorrhage is a rare finding. Some patients have neurological signs and rarely hepatic or renal failure. The fever lasts for a week or longer and is followed by slow convalescence. In approximately 20% of patients, there is a second phase of neurological illness with return of fever, headache, stiff neck, tremor, and an increase of white blood cells in the cerebrospinal fluid. The case fatality rate is 5% to 10%. Fatalities are secondary to encephalitis, hemorrhage, pneumonia, and hepatic or renal failure.

The 1957 epidemic and subsequent outbreaks were characteristically in settings where forest was being cut for lumber or plantations. These intrusions into the forest disrupted the ecology and exposed humans to the bites of *Haemaphysalis spinigera* ticks, the principal vector of the virus. They also allowed for grazing of cattle, the preferred host for the adult tick, thus rapidly increasing the numbers of ticks in the forest as cattle entered. A further factor was the spread of vegetation, such as the Lantana bush, which formed dense cover for small mammals, birds, deer, and cattle at the edge of the forest.

It was originally thought that KFD virus might have been introduced by birds or ticks on birds from other parts of Asia to initiate the 1957 epidemic. Large numbers of migrating birds were examined

with no evidence that they transported KFD virus. The conventional wisdom now favors existence of a cryptic focus that was lying in wait for humans as they disrupted the forest ecology (Varma, 2001).

Outbreaks have since occurred in 1972, 1973, 1975, 1983, 1988–1989, 1990–1992, 2000, and 2003. The original focus covered a few hundred square kilometeres. By 2003 the disease covered 5,000 square kilometers. Some of the outbreaks have been large. In 1983, for instance, 1555 cases and 150 deaths followed the clearing of 400 hectares for a Cashew tree plantation.

The main vectors of KFD virus are *H. spinigera* and *H. turturis*. These ticks are abundant in the forest. The immature stages are found on squirrels and other small forest mammals, as well as on ground-feeding birds. It is the nymphal stage that transmits KFD virus to people and monkeys. The common langur monkey and the Bonnet macaque are heavily parasitized by the nymphs and commonly are found dead in the forest. The monkeys develop high titer viremia and serve as amplifying hosts of the virus. The adult stage of the tick feeds mostly on cattle and other large mammals, leading to increased population numbers of the ticks, although cattle do not develop viremia of any significance. It is not clear how KFD virus is maintained between epidemic periods. The squirrel is a potential reservoir host, but it is distributed mainly at the forest fringe and thus seems unlikely to play a major role in virus maintenance.

An effective inactivated vaccine is available for KFD. The vaccine was used in 1990–1992 in a trial of 88,152 persons in the KFD enzootic area. Of 349 cases, 24 were in vaccinees and 325 in unvaccinated persons (Varma, 2001).

In 1995, a virus closely related to KFD virus was isolated in Jeddah, Saudi Arabia, from six sick butchers. Two of the six patients died. Four additional patients were diagnosed by immunoglobulin M ELISA or seroconversion by immunofluorescence assay. The disease resembled KFD in many aspects, including fever, headache, muscle and joint pains, vomiting, and leukopenia. Patients also had abnormal liver enzymes and elevated blood urea nitrogen. One patient had encephalitic signs, and another had purpura and gastrointestinal bleeding (Zaki, 1997).

The virus was named Alkhurma virus, and its sequence indicated that it was a subtype of KFD virus (Charrel *et al.*, 2001).

C. Omsk Hemorrhagic Fever Virus

Virus was isolated in 1947 from blood of a 7-year-old boy who had fever, leukopenia, and a hemorrhagic syndrome. This was in the

setting of an outbreak in the Omsk Oblast of eastern Siberia that the previous year had afflicted 623 cases with four deaths. The Omsk hemorrhagic fever virus was found to be new but very closely related to Far Eastern tick-borne encephalitis virus (Karabatsos, 1985).

Smaller outbreaks with similar hemorrhagic fever cases had been observed in 1941, 1943, 1944, and 1945 in the northern forest steppe with many lakes and patches of birch-aspen forest Many of the cases had direct contact with the muskrat, *Odatra zibethicus*. These animals were introduced from North America during the 1930s and successfully established themselves in the Omsk and Novosibirsk Oblasts. The animals were hunted both for food during wartime periods and for their valuable fur. It is widely believed that the muskrat was responsible for amplifying the Omsk hemorrhagic fever virus with consequent epidemics. The narrow-skulled vole, *Microtus stenocranius gregalis*, has also yielded the virus. The prevalence of the disease dramatically decreased starting in 1951 coincident with a decline in the *Dermacentor* population. For several years during the 1960s and 1970s, there were no reported human cases, although active foci of muskrat transmission still exist (Gavrilovskaya, 2001).

The disease has an incubation period of 2 to 4 days and is characterized by fever, headache, malaise, conjunctival injection, arthralgia, myalgia, stiff neck, rash, and leukopenia. Pneumonia is a common complication. The white blood cell count is low, which helps to differentiate this illness from bacterial pneumonia. Hemorrhagic manifestations are nearly universal. There may be pulmonary and uterine bleeding, petechiae, conjunctival hemorrhage, and epistaxis. In about a third of cases, after an afebrile period of 10 to 18 days, there is a second episode of fever accompanied by return of a milder illness than the first phase. The convalescent period lasts 14 to 28 days and may be associated with loss of hair. The case-fatality rate is about 1% (Gavrilovskaya, 2001).

People are infected by the bite of *Dermacentor reticulatus* ticks from which the virus is frequently isolated. Virus has also been isolated sporadically from *D. marginatus, D. silvarum, I. persulcatus*, and *H. concinna* ticks. Omsk hemorrhagic fever virus may also be waterborne. Muskrats die with a hemorrhagic illness and are drawn to the lake margins, presumably because of thirst. Their bodies freeze in the ice, and the virus can be recovered from the water the next spring. How much this contributes to the survival of the virus over winter and the initiation of new infections in muskrats and humans the next season is not known (Kharitonova and Leonov, 1985). The illness has two peaks, spring and fall, corresponding to the prevalence of

Dermacentor ticks. Person-to-person transmission is not reported, but laboratory infections are common, presumably because of aerosol transmission.

D. Negishi Virus

In 1948 a flavivirus was isolated from the cerebrospinal fluid of a 6-year-old boy in Tokyo, Japan (Karabatsos, 1985). The child died. The virus was isolated from a second fatal case during the same outbreak in Tokyo, but Negishi virus has not been detected again in Japan since 1948. Other fatal cases during the 1948 epidemic were identified as Japanese encephalitis virus. Negishi virus is very closely related to louping ill virus, a member of the tick-borne encephalitis complex, and its presence in Tokyo during 1948 is not readily explained. The virus was subsequently recovered from *I. ricinus* ticks in southeastern Russia (Lvov, 1993). Nothing more is known about its epidemiology.

V. A Flavivirus Common to North America and Asia

A. Powassan Virus

Powassan virus was isolated postmortem from the brain of a 5-year-old boy from Powassan, Ontario, Canada, in September 1958. He presented with acute encephalitis with headache, fever, and spastic hemiplegia (Mclean and Donohue, 1959). Powassan encephalitis is a rare disease. Only thirty-one cases were reported in North America between 1958 and 2001. There were seven deaths, and more than half of the survivors had sequelae.

Most of the cases in North America have occurred in Ontario, Canada, and New York, Maine, and Vermont in the United States. The virus is also endemic in Primorye and Khabarovsky territories in Far Eastern Russia where it was isolated twice from patients. In a series of 11 human infections in Russia, the incubation was from 3 to 11 days, and the clinical syndromes ranged from febrile disease to meningoencephalitis (Lvov, 1993).

The reservoir of Powassan virus includes ticks and small mammals. *Ixodes cookei, I. marxi*, and *I. scapularis* are implicated as vectors in North America (McLean, 2001). In Russia, *Haemaphysalis* spp., *D. sylvarum*, and *I. persulcatus* ticks, as well as variety of mosquitoes, are reported to harbor the virus (Lvov, 1993). In laboratory experiments both in North American ticks (*I. scapularis*) and Russian ticks,

Powassan virus was shown to be transmitted transtadially and transovarially. The transmission period in nature is between May and October.

In North America the principal vertebrate host is the groundhog, *Marmota monax*. The American red squirrel, *Tamiasciurus hudsonicus*, and white-footed mouse, *Peromyscus leucopus*, have also been implicated. In Russia the vertebrate hosts most commonly infected are *Apodemus agrarius, A. peninsulae*, and *Microtus fortis*.

Powassan virus causes a necrotizing encephalitis with perivascular cuffing of the cerebral vessels and an inflammatory response focally surrounding necrotic neurons (Mclean and Donohue, 1959). Patients present with fever and headache and may progress in 1 to 3 days to gradual loss of consciousness. Signs of encephalitis, such as tremors, hemiplegia, stiff neck, and convulsions, are prominent. The case-fatality rate is between 20% and 25%. More than half of the survivors are left with sequelae such as weakness, hemiplegia, or memory loss.

The cerebrospinal fluid (CSF) examination reveals increased mononuclear cells and protein. The diagnosis can be made with reverse transcriptase-polymerase chain reaction of CSF and brain or immunoglobulin M capture ELISA of CSF or serum. In North America, the incursion of West Nile virus into the same geographical area as Powassan virus led to the recognition of Powassan encephalitis by the laboratory in cases that were negative for West Nile encephalitis and where the physician had not requested a test for Powassan encephalitis (CDC, 2001).

There is no vaccine or treatment for Powassan encephalitis. Infection can be prevented by protective clothing and search for ticks on the body and their removal. The *Ixodes* ticks tend to be very small, however, and are often overlooked.

Nucleotide sequencing of North American strains of Powassan virus in two different laboratories has shown that there are two genetic variants (Beasley *et al.*, 2001; Ebel *et al.*, 2001). Strains of both clades cause human disease, but their existence may mean that the variants have different reservoirs.

VI. Other Flaviviruses of North America

A. Rio Bravo Virus

A virus was isolated from a Mexican free-tailed bat, *Tadarida brasiliensis*, in 1954 during a search for new viruses in California. The bat was a member of a colony sequestered in the Rio Bravo School, Kern

County. Since then, multiple isolates have been made in other parts of California and in Texas from the same species. None of the bats appeared sick. Attempts to infect *Anopheles, Aedes*, and *Culex* mosquitoes by inoculation failed. Rio Bravo virus has infected one person under natural conditions and seven laboratory workers. Three of the laboratory infections were serious. Patients suffered from meningitis and inflammation of the ovaries or testes. It is not known how the virus is maintained in the bat population, and there is no evidence that mosquitoes are involved (Karabatsos, 1985; Theiler and Downs, 1973).

B. Modoc Virus

A presumptive serological diagnosis of Modoc meningitis was made in 1966 in a 10-year-old boy admitted to Kern General Hospital in California. The boy had a temperature of 103°F. The CSF was under increased pressure with a cell count of 450, 51% being lymphocytes. Paired sera showed a rise in hemagglutination-inhibition antibody to Modoc virus from 1:10 to 1:160. Modoc antigen gave a higher titer than did antigens of other flaviviruses known to be indigenous to California (Reeves, 1990). A second non-fatal Modoc virus infection of a laboratory worker is recorded but without details of the clinical syndrome (CDC/NIH, 1999).

Modoc virus was originally isolated from mammary tissue of a deer mouse, *Peromyscus maniculatus*, trapped in the Modoc National Forest in California. It was later isolated from deer mice in Alberta, Canada (Zarnke and Yuill, 1985). Attempts to replicate the virus in laboratory-inoculated *Aedes, Culex*, and *Anopheles* mosquitoes were not successful (Theiler and Downs, 1973). The mode of transmission is unknown, although experimentally infected deer mice harbor virus in their lungs up to 6 months, giving rise to the hypothesis that transmission from mouse to mouse is by the aerosol route (Reeves, 1990).

VII. Other Flaviviruses of South America

A. Bussuquara Virus

Bussuquara virus was isolated from the blood of a sentinel howler monkey, *Alouatta beelzebul*, infected in secondary forest near Belem, Brazil, in March of 1956. The monkey died and was found to have liver histopathology characteristic of yellow fever. The virus was later isolated in Panama from a febrile person who complained of headache

and myalgia. This isolate (Srihongse and Johnson, 1971) is the only Bussuquara virus recovered from humans. There is evidence by neutralization test that human infection also occurs in Colombia where Bussuquara virus was isolated from *Culex* spp. mosquitoes.

Extensive studies in the forests near Belem, Brazil implicated the Spiny rat, *Proechimys guyannensis*, as the vertebrate reservoir host. The dynamics of transmission were monitored in a capture-mark-release-recapture experiment over a 6-year period. The Spiny rat population was infected during the rainy season each year as monitored by serological conversion using the hemagglutination test. In addition, the virus was isolated from Spiny rat blood 29 times during this period. Other small mammals and birds did not seroconvert significantly. Studies of mosquitoes implicated *Culex (Melanoconion)* spp. On three occasions, wild-caught *Culex* transmitted virus when exposed to laboratory mice. In addition, isolates were made frequently in Brazil, Colombia, and Panama from forest-inhabiting *Culex*.

B. Ilheus Virus

Ilheus virus was one of the earliest isolated flaviviruses. It was recovered from a pool of *Ochlerotatus* and *Psorophora* mosquitoes near Ilheus, Bahia, Brazil. The mosquitoes were captured in old secondary growth and virgin forest and inoculated into a rhesus monkey that subsequently developed viremia and fever. Judging from neutralization test results, Ilheus virus infects large numbers of persons throughout northern South America, Trinidad, and Central America. In contrast, disease in humans is rarely recognized. The virus has been isolated in eight persons in Brazil, three in Trinidad, and one each in Panama, Argentina, and Colombia (Barrett, 2001; Karabatsos, 1985; Nassar, 1997).

The clinical syndrome is characterized by sudden onset of fever, myalgia, headache, and malaise. Two patients had suspected encephalitis. Six cancer patients in New York City were inoculated with Ilheus virus experimentally; one developed mild encephalitis. There have been no fatalities. Ilheus virus is distinct from Rocio virus but closely related.

The virus is transmitted by mosquitoes. The mosquito most commonly found infected is *Psorophora ferox*, a forest ground–pool breeder. Isolates have also been obtained from *Psorophora albipes, Ps. lutzii, Ochlerotatus serratus, O. fulvus, O. leucocelaenus, Sabethes chloropterus, O. scapularis, Culex nigripalpus, Haemagogus spegazzinii falco*, and *Trichoprosopon* spp.

Studies in Trinidad and Panama implicated a variety of birds as hosts for Ilheus virus. Virus was isolated from birds of the genera *Crotophaga, Tachyphonus, Sporophila, Elaenia, Saltator, Florida*, and *Ramphastos*. Monkeys in the Amazon region and Trinidad have neutralizing antibody, and a sentinel monkey exposed in the forest near Belem, Brazil became viremic.

C. Rocio Virus

In March 1975 a remarkable epidemic of encephalitis was reported from a coastal plain region of São Paulo State, Brazil. A newly recognized flavivirus was isolated in December from cerebellar and spinal cord tissues of a 39-year-old male. The virus was later shown to be closely related to Ilheus virus and was named Rocio virus (Iversson, 1989).

The epidemic continued from 1975 into 1977 and involved 971 cases. The clinical syndrome was recorded in 234 patients with encephalitis. Onset was sudden with an incubation period of 1 to 2 weeks. Nearly all patients had fever and headache. Gastrointestinal manifestations including nausea, vomiting, abdominal distension, and loss of appetite were common. Patients had altered consciousness, pathological muscle weakness, altered deep tendon reflexes, and a striking loss of equilibrium. About 20% of encephalitis cases were left with sequelae (Karabatsos, 1985; Mitchell, 2001). The CSF had increased numbers of white blood cells and elevated protein. The case-fatality rate was 10%, but in children under 1 year of age it was 31%. In persons over 60 years of age, it was 28%.

Rocio encephalitis attack rates were higher in males, and usually there was an association with exposure in the forest. The virus was isolated once from forest *Psorophora ferox*; in addition, *O. scapularis* and *O. serratus* were noted to be abundant in the epidemic forest zone. Laboratory studies indicated that *P. ferox* and *O. scapularis* were the more efficient vectors, and these two species are thus likely to be important for transmission during epidemics.

The vertebrate hosts of Rocio virus in the Brazilian forests are not known. The virus was isolated on one occasion from a Rufous-collared sparrow, *Zonotrichia capensis*, in the epidemic zone. It is thus possible that birds are involved in the transmission cycle. There has been no evidence that Rocio virus is still active in São Paulo State since 1978. Thus, further elucidation of the transmission cycle will need to wait until the disease appears again.

VIII. Arboviruses of Australia and New Guinea

A. Edge Hill Virus

Edge Hill virus was discovered in 1961 in *Oc. vigilax* mosquitoes during a study of arboviruses at Edge Hill, near Cairns, North Queensland, Australia. The virus has since been recovered on multiple occasions from the same mosquito species and much less frequently from other species. It has also been recovered from mosquitoes in New South Wales. Wallabies (*Wallabia rufogrisea* and *W. elegans*) in Southeast Queensland have neutralizing antibody to Edge Hill virus, which is evidence but not proof that they are involved in the transmission cycle. A single, probable human infection was characterized by myalgia, arthritis, and muscle fatigue (Aaskov *et al.*, 1993). The infection was self-limited.

B. Kokobera Virus

Kokobera virus was initially recovered in 1960 from *Culex annulirostris* mosquitoes during a search for arboviruses at the Mitchell River Mission, Kowanyama, northern Queensland. The virus has since been isolated from *O. vigilax* and *O. camptorhynchus*. It is not known which of these species is important in the transmission cycle of the virus. The vertebrate host is also not known. Kokobera virus exists in Australia in multiple clades, which may indicate that the vertebrate host has a relatively small home range (Mackenzie, 2001a).

The evidence for human disease is based on serological diagnosis. Three cases were reported (Boughton, 1986) of acute polyarticular disease with fever, headache, and lethargy. One patient exhibited a macular pruritic rash that lasted 4 days. The joint manifestations persisted at least 5 months, but the disease was not life-threatening.

C. Sepik Virus

Sepik virus was possibly associated with human disease in New Guinea. The patient had a febrile illness with headache. The evidence consists of high titer neutralizing antibody. Sepik virus was initially isolated in 1966 from *Mansonia septempunctata* mosquitoes in the Sepik district of New Guinea. It was also isolated in the same region from *Ficalba* spp. and *Armigeres* spp. mosquitoes. The virus has yet to be associated with a forest vertebrate host. This virus is closely related to Wesselsbron virus, which has considerable human and domestic animal disease potential (Karabatsos, 1985; Mackenzie, 2001b).

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VACCINES AND ANTIVIRAL AGENTS

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FORMALIN-INACTIVATED WHOLE VIRUS AND RECOMBINANT SUBUNIT FLAVIVIRUS VACCINES

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The Flaviviridae is a family of arthropod-borne, enveloped, RNA viruses that contain important human pathogens such as yellow fever (YF), Japanese encephalitis (JE), tick-borne encephalitis (TBE), West Nile (WN), and the dengue (DEN) viruses. Vaccination is the most effective means of disease prevention for these viral infections. A live-attenuated vaccine for YF, and inactivated vaccines for JE and TBE have significantly reduced the incidence of disease for these viruses, while licensed vaccines for DEN and WN are still lacking despite a significant disease burden associated with these infections. This review focuses on inactivated and recombinant subunit vaccines (non-replicating protein vaccines) in various stages of laboratory development and human testing. A purified, inactivated vaccine (PIV) candidate for DEN will soon be evaluated in a phase 1 clinical trial, and a second-generation JE PIV produced using similar technology has advanced to phase 2/3 trials. The inactivated TBE vaccine used successfully in Europe for almost 30 years continues to be improved by additional purification, new stabilizers, an adjuvant, and better immunization schedules. The recent development of an inactivated WN

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vaccine for domestic animals demonstrates the possibility of producing a similar vaccine for human use. Advances in flavivirus gene expression technology have led to the production of several recombinant subunit antigen vaccine candidates in a variety of expression systems. Some of these vaccines have shown sufficient promise in animal models to be considered as candidates for evaluation in clinical trials. Feasibility of non-replicating flavivirus vaccines has been clearly demonstrated and further development is now warranted.

I. Introduction

The Flaviviridae is a family of arthropod-borne viruses (formerly classified as group B arboviruses), which infect mammalian hosts, including man, via infected mosquito or tick vectors (Westaway *et al.*, 1985; for a review, see Monath, 1990, 1999). There are over 70 members of the flavivirus family; these viruses are widespread and can be found in most inhabited regions of the world. Flaviviruses share a similar physicochemical composition, antigenic structure, genome organization, and replication scheme (reviewed by Chambers, 1990). They are small, enveloped viruses about 50 nm in diameter, with a spherical nucleocapsid that contains a single-stranded, positive-sense RNA genome approximately 11 kilobases in length. The genome encodes 11 proteins: four structural proteins: capsid (C), membrane (M) and its precursor premembrane (prM), and envelope (E); and seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. The structural E glycoprotein is the major virion envelope protein; it possesses domains for attachment to and fusion with host cells and contains epitopes that elicit virus-neutralizing antibodies important for immunity and protection. Although all flaviviruses are closely related genetically and antigenically, individual viruses are also antigenically distinct (Calisher *et al.*, 1989). Therefore, infection with one flavivirus does not result in significant cross-protection against other flaviviruses. This complicates vaccine development, especially for the dengue viruses where there are four different serotypes (see subsequent discussion).

Among the most medically important flaviviruses are yellow fever (YF, the family prototype), dengue (DEN), Japanese encephalitis (JE), tick-borne encephalitis (TBE), and West Nile (WN) viruses. The first flavivirus vaccine was developed over 60 years ago for YF (Theiler and Smith, 1937). This vaccine, made from the attenuated YF 17D strain and its derivatives, remains one of the safest and most effective

vaccines ever developed. The goal of approaching this high standard has led to the application of several different technologies for making vaccines for other members of the family Flaviviradae.

This review will focus on inactivated virus and recombinant subunit vaccines, which belong to the general category of non-replicating protein (NRP) vaccines, while other types of vaccines, such as live-attenuated vaccines (LAV), will be covered elsewhere in this volume (for additional reviews see Barrett, 2001; Brandt, 1990; Kinney and Huang, 2001; Putnak, 1994). NRP vaccines, especially when formulated with adjuvants, can be very effective at eliciting high titers of virus neutralizing antibodies, which are thought to be the primary correlate of protection against flaviviruses. Such vaccines are usually less reactogenic than LAV; however, they may require more frequent booster vaccinations to maintain a state of protective immunity. They may not be as effective at generating cytotoxic T-lymphocyte (CTL) and memory T-cell responses as vaccines that are processed through intracellular pathways. However, it should be possible to overcome these drawbacks through the use of newer and better adjuvants and immunomodulators that are able to activate specific arms of the immune system. NRP vaccines might also be used in prime-boost vaccination strategies, combined with other immunogens that induce effective cell-mediated immune (CMI) responses, but not high titered antibodies. For example, LAV or DNA vaccines might be used before or after vaccination with NRP vaccines.

II. Inactivated Whole Virus Vaccines

Inactivated virus vaccines require a culture system, either small animal or cell culture, for producing virus in sufficient quantity, which is then purified, inactivated, and formulated with adjuvants. As long as the inactivated virus retains its immunogenicity, the resulting vaccine should be safe, stable, and provide adequate protection. Unlike recombinant vaccines, a thorough understanding of viral molecular biology is not required to develop this type of vaccine. Disadvantages of inactivated vaccines may be their relatively greater production cost, and concerns stemming from the fact that the immune responses that such vaccines elicit may be qualitatively different from those elicited by vaccines that are processed through intracellular pathways. Even so, the large number of successful inactivated vaccines would seem to validate this approach. Table I summarizes the status of current approaches to whole virus-inactivated flavivirus vaccines.

TABLE I

WHOLE VIRUS–INACTIVATED FLAVIVIRUS VACCINES AND VACCINE CANDIDATES

Virus (cell substrate); Institution	Purification Process	Animal Immunogenicity/ Protection	Human Immunogenicity/ Protection	Clinical Status	References
DEN-2, S16803(Vero cells) WRAIR, US	Protamine sulfate, ultrafiltration, ultracentrifugation	N ab in mice, NHP Protective in mice, NHP	N/A	Phase 1 pending	Putnak *et al.*, 1996a,b
JE Nakayama and Beijing (mouse brain); BIKEN, Aventis* Japan, France	Protamine sulfate, ultracentrifugation	N ab in mice, NHP Protective in mice, NHP	77%–100% seroconversion; 91% protection	Licensed in U.S., Japan	Takaku *et al.*, 1968, 1971; Hoke *et al.*, 1988; Nimmannitya *et al.*, 1995; Defraites *et al.*, 1999; Poland *et al.*, 1990; Gambel *et al.*, 1995; Andersen and Ronne, 1991; Ruff *et al.*, 1991; Sakaguchi *et al.*, 1997, 1998; Plesner and Ronne, 1997
JE, SA_{14}-14-2 (Vero cells); WRAIR, US	Protamine sulfate, ultrafiltration, ultracentrifugation	N ab in mice Protective in mice	Immunogenic	Phase 2/3 planned	Srivastava *et al.*, 2000; Kanesa-thasan, personal com
JE, P3 (PHK cells); PRC	Not purified	N/A	60–70% seroconversion; 84–95% protection	Licensed in China	Ao *et al.*, 1983; Tsai *et al.*, 1999
JE, P3 (Vero cells); NVSI, PRC	Protamine sulfate, ultracentrifugation	N/A	95% seroconversion	Phase 2	Ding *et al.*, 1998

JE, Beijing-1 (Vero cells); BIKEN, Japan	Protamine sulfate, ultracentrifugation	Immunogenic and protective in mice	100% seroconversion in JE-immune subjects	Phase 1	Minutes of WHO Steering Committee on DEN and JE Vaccines, Apr. 2002
JE, Beijing-1 (Vero cells); Chemo-Sero Therapeutic Institute, Japan	Ultracentrifugation and column chromatography	N/A	100% seroconversion in JE non-immune subjects	Phase 1	Minutes of WHO Steering Committee on DEN and JE Vaccines, Apr. 2002
JE, P3 (Vero cells); Aventis-Pasteur, France	N/A	N/A	N/A	Phase 1 discontinued	N/A
TBE (CEF cells); Baxter AG, Austria	Ultracentrifugation	N/A	N/A	Licensed in Europe	Kunz *et al.*, 1992
WN (cell culture); Fort Dodge Animal Health, U.S.	N/A	N/A	N/A	Veterinary license (U.S.)	N/A
WN (mouse brain)	Not purified	Immunogenic in geese; 94% protection	N/A	N/A	Malkinson *et al.*, 2002

PHK, primary hamster kidney; CEF, chick embryo fibroblast; N/A, data not available; NHP, non-human primates; N ab, neutralizing antibody; PRC, People's Republic of China; WRAIR, Walter Reed Army Institute of Research.

*Also produced in S. Korea, Thailand, Taiwan, and Vietnam using similar technology.

A. Dengue Virus

Dengue (DEN) fever and related diseases, DEN hemorrhagic fever (DHF) and DEN shock syndrome (DSS), are caused by four serologically distinct viruses: DEN-1, DEN-2, DEN-3, and DEN-4. DEN has the highest incidence among all flavivirus-related diseases. It is endemic in the tropics and subtropics, where over 2 billion people are at risk and an estimated 100 million new infections may occur annually (for reviews, see Gubler, 1998; Henchal and Putnak, 1990). Primary DEN infection usually causes a flu-like illness characterized by 3 to 7 days of fever, headache, body ache and rash, which can be severe but not usually life-threatening. More severe diseases, DHF and DSS, are rare but can lead to death if untreated. The risk factors for DHF appear to be both virus- and host-related, but secondary dengue infection appears to pose the greatest risk. It has been postulated that immune enhancement of infection by cross-reactive, non-neutralizing antibodies plays a major role in DHF (Halstead, 1970; Sangawhiba *et al.*, 1984). This makes the development of a vaccine for DEN difficult because one must confer long-lasting protection simultaneously against all four serotypes to reduce the risk of DHF.

Attempts to develop inactivated vaccines for DEN began over 70 years ago, when phenol, formalin, or ox bile were used to inactivate DEN viruses in infectious mosquito pools or viremic plasma (Blanc and Caminopetros, 1929; Simmons *et al.*, 1931). Human volunteers were vaccinated with these crude vaccines and challenged a short time later. In one study using ox bile-inactivated virus vaccine, the investigators reported protection against disease following DEN challenge (Blanc and Caminopetros, 1929). Until recently, there has been little interest to develop inactivated DEN vaccines, mainly because of the widely held belief that DEN viruses cannot be propagated to sufficiently high titers and inactivated without loss of immunogenicity. However, work carried out by researchers at the Walter Reed Army Institute of Research (WRAIR) demonstrated that it is feasible to produce an inactivated DEN vaccine. Initial studies using DEN-2 virus established the basic production process: virus was propagated in fetal rhesus lung (FRhL) diploid cell cultures, harvested from the culture supernatants, filtered, and inactivated at 22 °C with 0.05% formalin (Dubois, Doria, Ph.D. Thesis, Catholic University of America, 1980). This unpurified vaccine induced anti-DEN neutralizing antibodies in mice and protected vaccinated animals from lethal virus challenge. This work was extended by Putnak *et al.* (1996a, 1996b), who used low-passage strains of all four serotypes of DEN virus adapted to grow to high titers

(ca. 10^7 plaque-forming units/mL) in certified Vero cell cultures (Montagnon *et al.*, 1999) to prepare a prototype DEN-2 purified-inactivated vaccine (PIV). Virus was harvested from serum-free supernatant culture fluid, concentrated, highly purified on sucrose gradients, and inactivated with 0.05% formalin for 10 days at 22 °C (Putnak *et al.*, 1996a). This vaccine contained viral structural proteins E, prM and M, and trace amounts of NS1. Mice immunized with two 1.5-ug doses of vaccine adjuvanted with 0.01% alum given one month apart made high-titered virus neutralizing and hemagglutination-inhibiting (HAI) antibodies. Groups of rhesus monkeys that received three inoculations with doses ranging from 0.25 to 75 μg, administered at days 0, 28, and 6 months also made neutralizing antibodies in a dose-dependent fashion. Those groups that received doses of 7.5 μg or greater developed geometric mean neutralizing antibody titers (GMT) of 1:80 or greater and demonstrated significant protection against virus challenge 2 months after vaccination. A new DEN-2 PIV lot produced in serum-free medium under cGMP has proven satisfactory in initial preclinical safety evaluations and is scheduled for evaluation in a phase 1 clinical trial in 2002 (K. Eckels, personal communication).

B. Japanese Encephalitis Virus

JE infection, although usually subclinical, has the potential to cause fatal encephalitis and life-long neurologic problems in survivors. Before the widespread use of inactivated JE vaccine in Japan and inactivated and live-attenuated vaccine in China, thousands of cases of JE occurred each year in those countries (reviewed by Burke, 1988; Vaughn and Hoke, 1992). JE remains a serious health threat in many other countries where vaccination is not routinely practiced.

Currently, there is only one JE vaccine licensed by the United States Food and Drug Administration (FDA) for use in the United States: a purified, inactivated vaccine developed at the Research Foundation for Microbial Diseases of Osaka University (BIKEN) in Japan. This vaccine is made from a wild-type strain of the JE virus (either Nakayama or Beijing) propagated in the brains of suckling mice, formalin-inactivated, purified, and formulated with a gelatin stabilizer (Takaku *et al.*, 1968, 1971). It has been in continuous use in Japan for over 30 years, where it has proven to be relatively safe and effective. A large field efficacy trial sponsored by the United States Army was performed in Thailand to compare monovalent BIKEN vaccine with a bivalent version made from Nakayama and Beijing-1 strain viruses

(Hoke *et al.*, 1988). This study was placebo-controlled, masked, and randomized and followed school-age children in Northern Thailand given two doses of vaccine a week apart. There were nearly 22,000 children in each group and the efficacy of both monovalent and bivalent vaccines was 91%. There were no major side effects or serious adverse reactions. Minor side effects and reactions such as headache, sore arm, rash, and swelling were similar to what was observed in the placebo group (tetanus toxoid) and occurred in less than 1% of vaccinees. In a subsequent study carried out in the United States in 1990, groups of volunteers were inoculated with either of three consecutively manufactured lots of BIKEN vaccine. Symptoms were generally limited to mild local reactions, and mean anti-JE virus neutralizing antibody titers were similar among groups (Defraites *et al.*, 1999). Data from these studies were used to obtain licensure of the BIKEN JE vaccine in the United States in 1993. Although this vaccine continues to be produced in Japan, it is now commercially distributed outside of Japan by Aventis-Pasteur under the trade name JE-VAX. Unfortunately, it is expensive and sees limited use in developing countries. Since 1986, there have been an increasing number of reports of adverse events, including allergic reactions, in persons receiving the mouse brain-derived JE vaccine (Andersen and Ronne, 1991; Ruff *et al.*, 1991; Sakaguchi *et al.*, 1997, 1998). In approximately one third of the cases, there was evidence for a predisposition to allergic reactions; however, there have been no reports that implicate specific allergens in the vaccine (e.g., the gelatin stabilizer or residual traces of mouse neural proteins).

There has been much interest to move away from mouse brain as a vaccine substrate and into mammalian cell cultures for developing new inactivated JE vaccines (Tsai, 2000). An inactivated vaccine made from the P3 strain of JE virus grown in primary hamster kidney cells is used in China, although lately it has been largely supplanted by the SA_{14}-14-2 live-attenuated vaccine, which is cheaper to produce and probably more efficacious (Ao *et al.*, 1983). The SA_{14}-14-2 virus, after additional passages in primary dog kidney (PDK) and Vero cell cultures, was used to make a second-generation purified-inactivated JE vaccine (Srivastava *et al.*, 2000). The production of this vaccine followed the same process developed earlier for DEN-2 PIV (see previous discussion). The JE PIV was tested for potency in mice, where a dose of 4 ng resulted in a 50% seroconversion rate for neutralizing antibodies, and a dose of 2.6 ng protected 50% of vaccinated animals against lethal challenge (Srivastava *et al.*, 2000). JE PIV has recently been tested in a phase 1 clinical trial at doses of 0.4 and 2 μg, where it

was found to be safe and immunogenic (N. Kanesa-thasan, personal communication). A second lot of the vaccine is currently being tested in a phase 2 clinical trial. Testing for protective efficacy in the rhesus monkey model, where animals will be challenged with a virulent strain of JE virus is planned. Other groups are also working to produce purified, inactivated JE vaccines in Vero cells (Ding *et al.*, 1998).

Concerns have frequently been raised that inactivated JE vaccines produced from a single strain of virus (e.g., Beijing or Nakayama alone) may not confer adequate cross-protection against all other epidemic JE strains. However, while there is demonstrated antigenic variation among different strains of JE virus (Ali and Igarashi, 1997), these viruses are genetically well conserved within the E gene region (Tsarev *et al.*, 2000). Not surprisingly, they were capable of generating cross-neutralizing antibodies, which should confer adequate cross-protection even though the titers against heterologous strains were lower than those against the homologous (vaccine) strain (Kurane and Takasaki, 2000).

C. Tick-Borne Encephalitis Virus

TBE, which causes a disease similar to Japanese encephalitis, posed a serious threat to Central and Eastern Europe before a vaccination program in 1973 (reviewed by Kunz, 1992). The first vaccine was made from inactivated mouse brain–derived virus, followed by an improved inactivated vaccine made from virus grown in chick embryo cells (Reviewed by Kunz, 1992). These first-generation vaccines were highly effective but also reactogenic, prompting continued development of more purified vaccines (Heinz and Kunz, 1977). In 1980, the development of a second generation TBE vaccine was reported (Heinz *et al.*, 1980), which used continuous-flow zonal ultracentrifugation for purification of formalin-inactivated virus. This vaccine, which was approximately 100-fold more pure than previous preparations, was highly immunogenic and appeared to be less reactogenic (Kunz *et al.*, 1980). Additional improvements were reported in 1990 with an inactivated vaccine made from TBE strain K23, purified by zonal centrifugation to a very high degree of purity, stabilized with degraded gelatin (polygeline) and adjuvanted with 0.2% aluminum hydroxide (Bock *et al.*, 1990). Clinical trials were conducted with this vaccine (Encepur) to test a 0 and 28-day immunization schedule and doses ranging from 0.03 to 3 μg. They demonstrated that the vaccine was reasonably well tolerated with a total of 10 adverse vaccination events including local reactions, headache, flu-like symptoms, and nausea reported by six of

56 volunteers. It was immunogenic in a dose-dependent fashion, eliciting geometric mean titers (GMT) of TBE neutralizing antibody that ranged from 1:49.8 (0.03 μg dose) to 1:3504 (3 μg dose). Subsequently, a larger clinical trial was carried out with three different dosages (1, 1.5, and 2 μg) and two immunization schedules: a conventional schedule of 0, 28, and 300 days; and an abbreviated schedule of 0, 7, and 21 days (Harabacz *et al.*, 1992). With both schedules, seroconversion rates measured by neutralizing antibody assays approached 100% and the antibody titers achieved were similar. However, those persons vaccinated according to the conventional schedule demonstrated a significant increase in their neutralizing antibody titers following a booster vaccination on day 300. The frequency of adverse events (both vaccine-related and unrelated) following primary vaccination was 37% for recipients of the conventional schedule and 46% for recipients of the abbreviated schedule. These decreased to 9% and 21%, respectively, following the second dose, and to 5% and 15% following the third dose. Side effects included headache, fever, and local reactions; however, these were reported to be generally mild and no serious adverse events were reported.

In the 1992 vaccination season, there were 1300 reports of suspected vaccine reactions with the Encepur TBE vaccine, including fever in some children, which prompted the development and clinical evaluation of a reduced dose, pediatric formulation (Girgsdies and Rosenkranz, 1996). The multicenter study enrolled 522 children, 18 months to 14 years of age, and 191 adults, 18 to 60 years of age. The participants were randomly assigned to either of three dosage groups, the full 1.5 μg conventional dose as well as 0.4 and 0.75 μg reduced doses. The recommended (abbreviated) vaccination schedule of 0, 7, and 21 days was used. The aim was to determine if the lower doses would be less reactogenic than the full dose while preserving adequate immunogenicity as measured with enzyme-linked immunosorbent assay and neutralizing antibody titers. The results showed that the antibody titers in those children who received the lower doses approximated titers in adults who received the full dose. However, this effect diminished with age and was not seen in children older than 12 years of age. Importantly, children who received the lower doses had an almost twofold lower frequency of fever than those who received the full dose.

D. West Nile Virus

Before 1999, West Nile (WN) virus disease was primarily a problem confined to the Middle East and Africa. In 1999, the first outbreak in the United States occurred in New York, possibly imported through

an infected bird. As of 2002, the virus has spread across the continental United States as far as California. Recently, an inactivated WN vaccine for horses was developed in the United States by Fort Dodge Animal Health, a division of Wyeth. This vaccine has recently received a 1-year license from the USDA. A prototype, formalin-inactivated WN vaccine produced in Israel from infected mouse brain was tested for control of WN disease in domestic geese (Malkinson *et al.*, 2002). This vaccine protected up to 94% of geese against lethal WN virus challenge following immunization with two doses. These results suggest that similar approaches can be used for developing an inactivated WN vaccine for human use.

III. Recombinant Subunit Virus Vaccines

Various gene expression systems have been used to produce immunogenic flavivirus proteins for use in recombinant subunit vaccines. This work has been facilitated over the past decade by advances in recombinant expression technology and in our understanding of the molecular biology of flaviviruses. Especially important were the cloning and sequencing of YF virus (Rice *et al.*, 1985), the production of the first flavivirus infectious cDNA clone (Rice *et al.*, 1989), and the determination of the crystal structure of the envelope (E) protein of TBE virus (Heinz *et al.*, 1991; Rey *et al.*, 1995). An understanding of the intracellular processing of flavivirus proteins and their secretion from cells has also played a key role in the successful development of recombinant subunit immunogens. Although much of this expression work was carried out using recombinant vaccinia, and is therefore outside the scope of this review, it deserves mention here. When the full-length E protein is expressed by itself in mammalian cells, it remains within the cell in a form that has relatively poor immunogenicity. However, when it is expressed together with prM (i.e., as a prM-E fusion protein), it is secreted as an extracellular, virus-like or subviral particle, which is highly immunogenic (Konishi *et al.*, 1992, 1993). The prM protein is thought to act as a chaperone for the E protein to prevent conformational changes from occurring inside the cell that are associated with membrane fusion (Guirakhoo *et al.*, 1989). These changes can alter the conformation of the E protein and render it less immunogenic. Interestingly, the E protein without its C-terminal hydrophobic membrane-anchoring domain (i.e., 80% E) is also secreted efficiently in a highly immunogenic, soluble form in the absence of prM (Men *et al.*, 1991). These findings have been exploited for the development of

flavivirus subunit vaccines (see subsequent discussion). Recombinant technology has also made possible the preparation of subunit antigens for nonstructural proteins NS1 and NS3, which have been shown to induce protective humoral and cell-mediated immune responses (Kurane *et al.*, 1991, 1998; Schlesinger *et al.*, 1987). Table II summarizes the development of recombinant vaccine candidates (subunit and extracellular particle) for flaviviruses.

A. Dengue Virus

Not surprisingly, *Escherichia coli* was the first expression system to be used for producing DEN immunogens, but the results proved to be generally disappointing. Although large amounts of viral proteins can be produced, the antigenicity and immunogenicity of the proteins were poor (reviewed in Putnak, 1994). One notable exception was the B-domain (i.e., domain III) at the C-terminus of the E protein, which can be expressed in *E. coli* in an immunogenic form. A peptide containing the C-terminal 204 amino acids of DEN-2 E protein and 65 amino acids from the N-terminus of NS1 fused to staphylococcal protein A and purified over a protein A affinity column, elicited high-titered neutralizing antibodies in mice and conferred excellent protection against challenge with live virus (Srivastava *et al.*, 1995). Whether or not the NS1 peptide region contributed to protection in this case is not known. Similar results were obtained in mice using the B-domain of DEN-2 E protein expressed as a fusion protein with the *E. coli* maltose-binding protein (Simmons *et al.*, 1998). One concern, however, is that small proteins such as this may contain too few epitopes for generating effective, long-lasting immunity, a conclusion that was supported by an experiment in rhesus monkeys. Animals that were vaccinated with the DEN-2 domain B/protein-A fusion protein made high-titered but very short-lived antibodies that were not protective against virus challenge (Putnak, unpublished results). Reports of successful expression in yeast also appear to be rare, except for one attempt to express DEN structural proteins C, prM and E by chromosomal integration in *Pichia pastoris*, which led to the production of 30 nm virus-like particles capable of inducing virus-neutralizing antibodies in rabbits (Sugrue *et al.*, 1997).

Recombinant baculovirus (*Autographica californica* nuclear polyhedrosis virus) has been used extensively for expressing viral genes in *Spodoptera frugiperda* (Sf9) cells to produce recombinant subunit immunogens (reviewed by Cochran *et al.*, 1987). Baculovirus-expressed DEN-1, DEN-2, and DEN-3 E proteins, truncated by varying amounts

at the C-terminus to remove the hydrophobic, membrane-anchoring domain, elicited low-titered neutralizing antibodies and conferred partial protection in mice against virus challenge (Delenda *et al.*, 1994a, 1994b; Feighny *et al.*, 1994; Putnak *et al.*, 1991). Another E protein construct, affinity purified using a C-terminal poly-His fusion peptide, also elicited neutralizing antibodies in mice, with isotypes similar to those elicited by inactivated virus. Vaccinated mice were protected against live virus challenge (Staropoli *et al.*, 1996, 1997). Similar results were reported for the full-length E protein of DEN-2, although it formed large extracellular aggregates (Kelly *et al.*, 2000). In mice, this particulate antigen induced mainly IgG subclass 1 antibodies, in contrast to live virus, which induced mainly IgG subclass 2 antibodies (Smucny *et al.*, 1995). Baculoviruses have also been used to express DEN nonstructural protein immunogens. A cell extract containing recombinant baculovirus-expressed DEN-4 NS1 protected mice from lethal DEN-4 infection (Lai *et al.*, 1989). Attempts to immunize non-human primates with baculovirus-expressed DEN antigens have met with only limited success. Rhesus monkeys vaccinated with crude cell extracts containing DEN-4 structural and nonstructural proteins, previously shown to immunize and protect mice (Zhang *et al.*, 1988), made antibodies detectable by EIA but not by virus neutralization or hemagglutination inhibition (HAI) assays (Eckels *et al.*, 1994). In these experiments, protection against viremia was seen in only one of three monkeys vaccinated with a lysate containing E protein and one of six animals vaccinated with a lysate containing C, M, E, NS1, and NS2A proteins. However, most of the vaccinated animals appear to have been primed, because they demonstrated accelerated HAI antibody responses following virus challenge. More highly purified baculovirus-expressed E antigen preparations also conferred little or no protection against virus challenge (Putnak, unpublished results; Velzing *et al.*, 1999).

Relatively little work has been reported using non–virus-vectored expression of flavivirus immunogens in mammalian cells. Recently, a CHO cell-derived line that is stably transformed with the DEN-2 prM-E gene region was developed (Konishi *et al.*, 2002). These cells continuously secrete DEN-2 E protein in the form of subviral, extracellular particles (EP). The amount of E antigen produced is similar to that produced in DEN-2 virus-infected Vero cells. The EP E antigen is also biochemically and antigenically equivalent to native E antigen. However, stable cellular transformation required the introduction of a mutation in the prM protein to inhibit the furin-catalyzed prM to M cleavage. This mutation prevents the formation of fusion-competent

TABLE II

RECOMBINANT FLAVIVIRUS (SUBUNIT AND EXTRACELLULAR PARTICLE) VACCINE CANDIDATES IN DEVELOPMENT

Virus (expression system)	Antigenic Composition	Animal Immunogenicity	Animal Protection	Human Immunogenicity/ Clinical Status	References
DEN (baculovirus/Sf9 cells)	NS1	N/A	mice	N/A	Lai *et al.*, 1989
DEN (baculovirus/Sf9 cells)	80% of E (secreted monomer)	N ab in mice, NHP	mice	N/A	Men *et al.*, 1991
DEN (baculovirus/Sf9 cells)	E truncated at the C terminus	N ab in mice	mice	N/A	Putnak *et al.*, 1991
DEN (baculovirus/Sf9 cells)	E; C-M-E-NS1	N ab mice	mice; partial protection in NHP	N/A	Eckels *et al.*, 1994
DEN (baculovirus/Sf9 cells)	E (fusion with poly-His)	N ab in mice	mice	N/A	Staropoli *et al.*, 1996, 1997
DEN (baculovirus/Sf9 cells)	100% E (intracellular particles)	N ab in mice	mice	N/A	Kelly *et al.*, 2000
DEN (CHO cells)	PrM-E (extracellular particles)	N ab in mice	N/A	N/A	Konishi *et al.*, 2002

DEN (yeast)	C-prM-E (extracellular particles)	N ab in rabbits	N/A	N/A	Sugrue *et al.*, 1997
DEN (*E. coli*)	C terminal E-NS1 (fusion with staph A)	N ab in mice	mice	N/A	Srivastava *et al.*, 1995
DEN (*E. coli*)	B-domain of E (fusion with protein-A or MBP)	N ab in mice, NHP	mice	N/A	Simmons *et al.*, 1998
JE (CHO cells)	PrM-E (extracellular particles)	N ab in mice	mice	N/A	Konishi *et al.*, 2001
JE (COS-1 cells)	PrM-E (extracellular particles)	N ab in mice	N/A	N/A	Hunt *et al.*, 2001
SLE (baculovirus/Sf9 cells)	PrM-E	N ab in mice	mice	N/A	Vanugopal *et al.*, 1995
TBE (COS cells)	E (extracellular particles)	N ab in mice	mice	N/A	Allison *et al.*, 1995; Heinz *et al.*, 1995
WN (*E. coli*)	E-thioredoxin or 80% E-MBP	N ab in mice	mice	N/A	Wang *et al.*, 2001

CHO, Chinese hamster ovary; MBP, maltose-binding protein; N/A, data not available; NHP, non-human primates; N ab, neutralizing antibody.

E dimers, which appear to be deleterious to the cells. Mice immunized at 2-week intervals with 100 or 400 ng of EP in Freund's adjuvant made DEN-2 neutralizing antibodies with $PRNT_{80}$ titers of 1:20 to 1:40 following a second dose. A non-lethal challenge was performed 2 weeks after vaccination by intraperitoneal inoculation of 10^6 pfu of live DEN-2 virus, whereupon a brisk, post-challenge anamnestic antibody response was observed, indicative of limited replication of the challenge virus. Although sterile immunity was not achieved, the authors argued that the observed anamnestic neutralizing antibody response is critical for protection against disease, based on the JE mouse model where there was a correlation between anti-JE anamnestic neutralizing antibody responses and protection against lethal challenge (Konishi *et al.*, 1998). However, because the diseases caused by DEN and JE viruses are essentially dissimilar, the mechanisms for protection might also be different.

B. Japanese Encephalitis Virus

Similar to DEN, there were early unsuccessful attempts to express immunogenic subunits for JE in *E. coli* (reviewed by Putnak, 1994). A significant breakthrough came with the observation that co-expression of JE prM and E genes in mammalian cells by recombinant vaccinia virus resulted in the secretion of virus-like particles that were similar in conformation and antigenicity to native virions (Konishi and Mason, 1993). When administered to mice as a subunit immunogen, these particles induced neutralizing antibodies, virus-specific memory T lymphocytes, and conferred protection against live virus challenge (Konishi *et al.*, 1992, 1997). The demonstration that prM is required for the correct processing and secretion of E protein is an important finding, and may explain why many earlier attempts to express E antigen alone in an immunogenic form were unsuccessful.

More recently, the same group developed a CHO cell line continuously expressing JE extracellular particles (EP) (Konishi *et al.*, 2001), similar to the cell line developed by these investigators for expressing DEN-2 EP (Konishi *et al.*, 2002). Mice immunized with two 1-μg doses of the JE EP developed $PRNT_{90}$ titers greater than or equal to 1:80, and a single 1-μg dose protected 50% of mice from lethal JE virus challenge. Similarly, Hunt *et al.* (2001) reported the development of a stably transformed COS-1 cell line producing a recombinant, particulate JE antigen. The recombinant antigen formed extracellular, 30-nm particles that contained E, prM and M proteins. The particles hemagglutinated like JE virions and could be purified on sucrose

gradients, banding at a density of 1.15 g/cc. Mice immunized with a single 1-μg dose of the recombinant antigen in Freund's incomplete adjuvant made anti-JE virus neutralizing antibodies, with $PRNT_{70}$ titers as high as 1:400.

Recombinant baculovirus has also been used to produce subunit immunogens for JE by expressing the genes for E, NS1, or a major portion of the polyprotein encoding C through NS3 (McCown *et al.*, 1990). When administered to mice in the form of unpurified cell extracts, a significant degree of protection against lethal virus challenge was observed in animals that received E and polyprotein-containing extracts. Protection appeared to correlate with the presence of low levels of virus neutralizing antibody in the vaccinated animals at the time of challenge.

C. Tick-Borne Encephalitis Virus

Given the availability of a licensed inactivated vaccine (see previous), progress to develop recombinant subunit TBE vaccine candidates has been limited. In one study several soluble and particulate TBE antigen preparations were prepared from purified whole virions and from recombinant E protein produced by expression in COS cells (Allison *et al.*, 1995; Heinz *et al.*, 1995). The subunit antigens and a purified-inactivated vaccine control adjuvanted with aluminum hydroxide were tested in mice for immunogenicity and protective efficacy. The highest titers of virus neutralizing and HAI antibodies and the best protection against virus challenge were seen with the purified-inactivated vaccine and with those subunit antigens that formed particles. In contrast, soluble subunit antigens elicited little or no antibody and failed to protect against challenge. The authors concluded that antigen conformation is important and that the recombinant subviral particles may be useful vaccine candidates.

D. St. Louis Encephalitis Virus

St. Louis encephalitis is an important mosquito-borne viral disease affecting North and South America, responsible for thousands of cases of disease and a number of deaths since it was first recognized over 60 years ago (Sabin *et al.*, 1943). There is no licensed vaccine; however, recombinant subunit immunogens produced from a variety of expression systems have been tested in animal models. As an example of a prime-boost strategy, good protective immunity was generated in mice vaccinated with a live vaccinia vector containing prM and E, followed by

boosting with recombinant baculovirus-expressed prM and E antigens (Venugopal *et al.*, 1995). Neutralizing antibody titers generated using this strategy were fivefold higher than those following vaccination with the individual vaccines. Protection against lethal SLE challenge also improved from 60% with the baculovirus recombinant subunit alone, and 80% with the vaccinia recombinant alone, to 100% with the combined, prime-boost vaccination strategy.

E. West Nile Virus

A recent publication by Wang *et al.* (2001) reports the expression of WN virus E protein in *E. coli* as a fusion protein with both thioredoxin and maltose binding protein. The E-MBP fusion protein contained an 80% E that was truncated on the C terminus to increase solubility and yield. The E-thioredoxin construct was immunogenic in mice and stimulated neutralizing antibodies after three doses. Mice immunized with E-MBP were protected against lethal challenge with WN virus. These results are encouraging and should be followed up with further animal experimentation.

IV. Summary and Conclusions

The success of JE and TBE purified-inactivated vaccines, which have greatly reduced the morbidity and mortality from infection with these viruses, offers hope for the development of safe and effective non-replicating vaccines for other flaviviruses, especially DEN. Tables I and II provide a summary of all the flavivirus vaccines currently reviewed, including those licensed as well as candidates still undergoing development and testing.

Improved methods for vaccine production, gene expression, and protein purification have led to increased interest in developing safer and more effective second-generation JE vaccines. Several purified-inactivated JE vaccine candidates produced in mammalian cells have already, or will soon enter phase 1/2 clinical trials (Proceedings of the WHO Flavivirus Vaccine Steering Committee, Geneva, 2001, 2002).

The technology for producing recombinant flavivirus immunogens has progressed significantly, with solid evidence that immunization of animals with virus-like particles elicits high-titered neutralizing antibodies that confer protection against virus challenge. Although all of the advantages and disadvantages of recombinant subunit versus purified-inactivated vaccines must still be weighed, similar technologies

(e.g., bioreactors and large-scale purification methods) will be applied for the production of both kinds of vaccines. The successful vaccine must not only be safe and effective, but also economically feasible and deliverable to the marketplace, including developing countries where the disease burden is greatest.

The successful development of new and improved adjuvants may be key to the success of future non-replicating protein vaccine candidates. Such adjuvants should confer increased immunogenicity at lower antigen doses and therefore give rise to more cost-effective and potentially safer vaccines. Unfortunately, the development of new, safe and effective adjuvants for prophylactic vaccines has progressed slowly for a number of reasons (for a review, see Gupta *et al*., 1993).

Clearly, a precedent for inactivated flavivirus vaccines has been established with the licensure of vaccines for JE and TBE. It remains a challenge for vaccine developers in both the public and private sectors to apply classical or molecular technologies for making new vaccines for DEN and other members of the Flaviviradae.

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ADVANCES IN VIRUS RESEARCH, VOL 61

EMPIRICALLY DERIVED LIVE-ATTENUATED VACCINES AGAINST DENGUE AND JAPANESE ENCEPHALITIS

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Although studies have been intensively conducted for over 50 years, there is still no commercial vaccine available against dengue disease. The lack of a suitable animal disease model, together with the lack of *in vitro* or *in vivo* markers of attenuation have been detrimental to the development of a tetravalent live-attenuated dengue vaccine. Historically, development of monovalent vaccine has substantiated the difficulty in maintaining a balance between a sufficiently attenuated vaccine and immunogenicity. Associating the four serotypes into a single dose (tetravalent vaccine) established the complex interactions among the four dengue serotypes. Here we review the major milestones in the long history of the dengue vaccine development, which recently led to phase 1 clinical studies in children in Thailand. Data from clinical studies with two dengue tetravalent live attenuated vaccines are examined and discussed. In contrast to dengue, vaccines against Japanese encephalitis (JE) virus played a major role in controlling JE disease in Asia. A lived-attenuated vaccine (SA14-14-2) was licensed in China in 1988. More than 100 million doses have been distributed there since. The vaccine appears safe, inducing >95% seroconversion after two doses. However, the vaccine is in need of improvement in terms of quality; there is a need to change the cell substrate (primary hamster kidney cells). This will be a significant step toward an international acceptance of this vaccine, which can play

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a major role in controlling the disease, particularly if the vaccine is integrated into the routine childhood immunization schedule.

I. Live-Attenuated Dengue Virus Vaccines

Dengue fever and dengue hemorrhagic fever are major health problems in tropical and subtropical regions. The increasing number of patients, the expansion of epidemic areas, and the increase in severe clinical manifestations have led to extensive research to develop dengue vaccines. Although vaccines against other flaviviruses have been developed, there is still no commercially available vaccine against dengue virus. An effective tetravalent vaccine that elicits long-term protective immunity against all four dengue serotypes is needed and is considered a priority by the World Health Organization (Chambers *et al.*, 1997). More than 50 years of intensive investigations have been spent conducting studies to develop a classical live-attenuated dengue virus vaccine. In this review the major milestones in the development of a vaccine which have led to phase 1 clinical studies in children in Thailand, using a live attenuated tetravalent vaccine produced on an industrial scale are reviewed.

A. *Mouse-Adapted Dengue Virus as a Live-Attenuated Vaccine*

Following isolation of dengue 1 (Hawaii strain) and dengue 2 (New Guinea strain) viruses during World War II, an attempt was made to develop a vaccine candidate by Sabin (1952) and Sabin and Schlesinger (1945). These investigators followed a strategy developed by Max Theiler who adapted yellow fever virus to the mouse by intracerebral inoculation. This resulted in the derivation of a yellow fever vaccine candidate called the French neurotropic virus (FNV). French workers used this vaccine extensively in West Africa until 1982. The mouse-adapted FNV played a major role in the fight against yellow fever. However, the incidence of adverse events including post-vaccination encephalitis were not rare. With time, the FNV vaccine was replaced by the safer 17D yellow fever vaccine developed at The Rockefeller Foundation by Theiler. Taking into consideration the biological properties of dengue virus, Sabin and Schlesinger (1945) succeeded in propagation of the Hawaii strain of dengue virus in mice. Tests with seventh, ninth, and 10th mouse passage virus indicated an important modification in its pathogenicity for humans leading to the selection of the mouse adapted dengue-1 virus as a vaccine candidate. The 15th

mouse-passage vaccine strain was tested in 16 volunteers at doses of 0.5 mL using three dilutions of 1:100, 1:1000, and 1:10,000. Fever and other systemic manifestations were significantly less marked than with earlier passage virus, but maculopapular rash of varying degrees occurred with some regularity. The modified infection was followed by development of solid virus specific neutralizing antibodies. The volunteers were protected when challenged by mosquitoes that had been infected with a virulent strain of dengue 1 virus. No difference in protection was observed among volunteers immunized with different doses of dengue-1 vaccine. Sabin (1952) concluded from these studies that the virus extract from a single mouse brain was sufficient to immunize 10,000 people.

The sixteenth passage mouse-adapted dengue 1 virus was adapted to grow in chick embryos after three passages and was given to four volunteers. The biologic properties of the vaccine were maintained at this further passage level, and a typical maculopapular rash without fever was observed.

Several Japanese investigators conducted extensive studies on dengue during World War II. Three mouse-adapted strains isolated by S. Hotta (1952) were identical to the dengue 1 Hawaii strains. Serial passage of the dengue 1 Mochizuchi strain by intracerebral (IC) inoculation of mice resulted in neuroadaptation, inducing 100% mortality in mice. The neuroadapted strain was inoculated into 5 human volunteers using a two-dose schedule over 1 week. The virus dose given was 10^4 mouse LD_{50}, and no abnormal signs were observed in the recipients. Immunized volunteers were inoculated intracutaneously with fresh serum from a dengue patient collected during the febrile stage. These volunteers were protected from dengue disease (Hotta, 1952). Complementary experiments were conducted in four additional volunteers; two of whom had a previous history of dengue infection. Two types of immune response were observed, which correlated with the immune status of the volunteer. Flavivirus-naïve volunteers developed neutralizing antibodies, which decreased rapidly after 11 weeks. On the other hand, pre-immune volunteers seroconverted and maintained a stable level of elevated neutralizing antibodies during the 18 weeks of observation (Hotta, 1952).

Wisseman *et al.* (1963) extended investigations with the mouse-adapted dengue-1 strain developed by Sabin. Four strains of the dengue 1 attenuated vaccine (MD1-4) derived from passage 18 were tested in 15 young adult volunteers. From results of this study, the MD-1 strain was selected for further investigation. Two groups of 10 volunteers were inoculated with 20,000 and 200,000 suckling mouse

LD_{50} of the virus. The vaccinees showed no clinical sign of infection and produced neutralizing antibodies (Wisseman *et al.*, 1963).

The MD1 dengue vaccine candidate is the only dengue vaccine tested under field conditions. During the 1963 Puerto Rico outbreak, a controlled study was conducted involving 1100 volunteers, 561 subjects were given 50–1000 LD_{50} of dengue-1 vaccine. The final report of this study was never published. Preliminary data indicated that no adverse reactions were observed (Bellanti *et al.*, 1966). Although this epidemic was associated with a dengue 3 virus, the incidence of disease among vaccinees was reduced to 39% of that in the control group. The observation that dengue 1 candidate vaccine provided partial protection against dengue 3 infection for 3 weeks to 85 days after vaccination may be attributed to the induction of heterologous antibodies (Bellanti *et al.*, 1966).

The genetic basis of attenuation of the Sabin-Wisseman vaccine has been partially elucidated. Ten amino acid differences separate the parental and mouse-adapted DEN2 vaccine strain developed by Sabin. Dissection of these mutations implicated a change from a negatively charged to a positively charged amino acid (Glu → Lys) at E126 within the hinge region of the E glycoprotein (Gualano *et al.*, 1998). A separate experiment involving neuroadaptation of DEN 1 virus by passage in mouse brain was associated with increased neurovirulence for mice and apoptotic cell death in neural cells but reduced apoptosis in human hepatocytes in culture (Duarte dos Santos *et al.*, 2000). These divergent effects on the induction of apoptosis in neural vs. hepatic cells were associated with a mutation in functional regions of the genome affecting virus replication and assembly: the hinge region at E196 (Met → Val), the interface between domains I and III at E365 (Val → Ile) as well as two mutations in the proximal stem-anchor (E405, Thr → Ile) and the NS3 helicase region.

Schlesinger *et al.* (1956) developed an attenuated dengue 2 (New Guinea B strain) virus by IC passage in suckling mice. The candidate vaccine was evaluated in volunteers either individually or in combination with dengue 1 or yellow fever 17D vaccines. Because of the small number of volunteers, it is difficult to evaluate this preliminary study. Inoculation of the mouse-adapted dengue 2 virus in 11 volunteers induced clinical reactions as mild as those seen following immunization with the attenuated dengue 1. Rash was the most consistent clinical manifestation. The combination of two or three viruses (dengue 1, dengue 2, and yellow fever) stimulated neutralizing antibodies against dengue 2 virus and depressed the antibody response to YF17D, suggesting that there was interference among the live-attenuated

vaccines given in combination. The interference in this experiment was attributed to the high dose of dengue 2 virus given (5.2–6.0-log mouse LD_{50}) compared to the commercial dose of yellow fever 17D (3.9 log mouse LD_{50}). No dose adjustment was performed to overcome this competitive effect. During this study it was established that individuals previously vaccinated against yellow fever reacted to immunization with attenuated dengue virus with a broadened anamnestic antibody response. Sera from the volunteers contained high levels of neutralizing antibodies against dengue 1, dengue 2, and yellow fever viruses. These findings suggest that immunization with yellow fever followed by injection of one or two dengue serotypes as a possible method of increasing vaccine immunogencity.

Further attempts to develop candidate vaccines using mouse brain–adapted dengue strains were abandoned due to the fear of neural tissue components, the presence of adventitious agents and the potential for neurovirulence in humans. From these early studies with mouse-adapted dengue viruses, the following conclusions can be made: a) Adaptation of the dengue viruses by IC inoculation in mice is followed by rapid modification of virus pathogenicity for humans; b) The clinical course following vaccination with mouse-adapted dengue viruses includes a mild dengue infection in which a maculopapular rash is the most consistent sign; c) Neutralizing antibodies appear around day 14, reaching peak titers between 3 and 4 weeks after inoculation. Little information has been obtained related to the antibody kinetics. Hotta (1952) demonstrated a rapid decrease in neutralizing antibody titers after 11 weeks in volunteers vaccinated with dengue 1. d) After vaccination, volunteers challenged with wild type dengue virus were uniformly protected. However, no long-term protection was evaluated using the immunization/challenge model. e) The combination of different attenuated flaviviruses in a vaccine formulation appears to induce interference. f) The possibility of sequential immunization with heterologous flaviviruses is controversial. Schlesinger *et al.* (1956) established that the vaccination with yellow fever followed by inoculation with the dengue vaccines is associated with a broadened immune response, suggesting that this approach could be considered as a vaccination strategy; however, no challenge with dengue wild type was performed to validate this hypothesis. In contrast, Wissemann *et al.* (1966) found that volunteers who had inapparent infection with JE virus and who subsequently received YF 17D vaccine developed a broad immune response and presented neutralizing antibodies against dengue 1. These individuals, however, were not protected against experimental dengue 1 infection.

B. Animal Models

There are no animal models that reproduce human dengue fever or dengue hemorrhagic fever. Mice are sensitive only to intracerebral inoculation and generally only after neuroadaptation. Monkeys inoculated with dengue viruses develop a viremia of 3 to 5 logs between days 2 to 6 post-inoculation, but do not develop any signs of dengue disease. Virus replication is followed by a strong immune response resulting in high levels of neutralizing antibodies and protection from virus challenge as measured by the lack of post-challenge viremia. Monkeys have been extensively used to evaluate the immunogenicity of dengue vaccine candidates to determine the best strategy for the development of a vaccine capable of protecting against each of the four dengue serotypes. Human epidemiologic data suggest symptomatic infection in man with a third or fourth dengue serotypes is unusual (Halstead *et al.*, 1967). It has been suggested that two sequential heterologous dengue virus immunizations may prevent disease upon subsequent challenge (Halstead *et al.*, 1973). Studies performed in rhesus monkeys by Halstead *et al.* (1973) showed that sequential dengue infections do not protect against virus challenge. Halstead and Palumbo (1973) provided convincing data relevant to developing a combined vaccine able to confer solid protective immunity against dengue (four dengue viruses into a single inoculum).

On the other hand, Prince (1968) showed that sequential immunization of spider monkeys with YF17D, dengue 2 and Japanese encephalitis viruses induced protection against challenges with wild type dengue 1, 3, or 4. In another experiment, Price *et al.* (1973) demonstrated that sequential immunization of monkeys with attenuated YF17D virus followed by inoculation with Langat virus or dengue 2 induced neutralizing antibodies against 20 flaviviruses tested. These vaccinated monkeys were protected against subcutaneous challenges with dengue serotypes 1–4.

These monkey studies suggest a significant difference in the immune response following sequential flavivirus inoculation. Protection against dengue infection can be achieved only when heterologous viruses (i.e. YF17D, JEV) are inoculated as the first two viruses. In contrast, sequential administration of two or three dengue virus serotypes is an unreliable method for producing broad cross-protection against dengue infection (Halstead *et al.*, 1973). Solid protection was only achieved by inoculation of all four dengue serotypes in a single or multiple sites (Halstead and Palumbo, 1973).

Monkeys have been used to identify phenotypic markers of dengue virus attenuation (Innis *et al.*, 1988). Biologic *in vivo* and *in vitro* tests such as plaque size, temperature sensitivity, and reduced replication in suckling mouse brain have been used for many years to evaluate possible markers of attenuation. However, multiple investigations using these parameters to compare live attenuated dengue vaccine candidates and their parental viruses failed to establish suitable markers of attenuation (Edelman *et al.*, 1994). Reduced duration of viremia in monkeys and more importantly significant reduction in the titer of the viremia appears to be the best marker of attenuation for evaluation of attenuated dengue viruses as vaccine candidate (Innis *et al.*, 1988).

C. Development of Live-Attenuated Dengue Vaccine in Cell Culture

Attenuation of dengue viruses as vaccine candidates has also been accomplished by passage in cell culture. Hotta *et al.* (1966) adapted the DEN-1 Mochizuki strain to monkey kidney or dog kidney cells in culture and determined that the new candidate vaccine maintained its immunogenicity for humans. Low titers of antibodies were detectable after a single dose inoculation and these increased after a boost at 2 to 4 months after the primary immunization. The immune response was not related to the dose of the vaccine. The same DEN-1 vaccine did not stimulate the production of neutralizing antibodies when administered as a formalin-inactivated virus (Hotta *et al.*, 1966).

The US military at the Walter Reed Army Institute of Research (WRAIR) used different strategies to select dengue vaccine candidates based on the cell substrate used to propagate and attenuate the viruses. Their strategy has been based on cloned (plaque purified) or uncloned viruses. Attenuation markers were *in vitro* (temperature sensitivity, plaque size) and *in vivo* (reduced neurovirulence for suckling mice, low or non-detectable viremia in monkeys after peripheral inoculation). In a double-blind, placebo-controlled study performed in 98 soldiers immunized with a dengue 2 vaccine candidate (PR-159/S-1), it was shown that previous immunization with YF 17D vaccine resulted in 90% seroconversion to dengue virus. Only 61% seroconversion was observed in volunteers not previously immunized with yellow fever vaccine (Bancroft *et al.*, 1984). The differences observed between yellow fever immune and yellow fever naive groups provides evidence suggesting that the presence of heterotypic cross-reacting antibody to flaviviruses enhanced dengue virus infection in humans resulting in greater antibody responses, and confirmed the earlier work of Schlesinger *et al.* (1956).

Subsequent phase I trials of the dengue type 4 candidate (Eckels *et al.*, 1984) and a dengue type 3 candidate (Innis *et al.*, 1988) were disappointing. The dengue 1 candidate vaccine (45 AZ5) obtained by plaque-to-plaque cloning has all of the *in vitro* and *in vivo* markers of attenuation. However, when the small plaque virus was inoculated into volunteers, a large plaque virus was isolated during the viremia and the recipients experience an unmodified dengue fever (McKee *et al.*, 1987).

Data obtained during clinical investigations at WRAIR in human volunteers identified the difficulty in maintaining a balance between vaccine attenuation and immunogenicity. The candidate vaccines were either overly attenuated or reactogenic in human volunteers. From these investigations, it was concluded that: a) no reliable *in vitro* markers for attenuation of dengue virus are known; b) no animal model can be used to predict attenuation for humans; and c) inoculation of human volunteers is the only way to determine the safety and immunogenicity of the vaccine (Edelman *et al.*, 1994).

D. Current Dengue Vaccine Development

Two tetravalent live-attenuated dengue vaccine candidates are currently under evaluation in humans by investigators at Aventis Pasteur/Mahidol University and WRAIR.

1. Aventis Pasteur/Mahidol University Tetravalent Live-Attenuated Dengue Vaccine

Four attenuated dengue virus strains were developed at Mahidol University, Bangkok, Thailand. Wild-type DEN 1-4 viruses were isolated from DF or DHF patients. DEN-1, DEN-2, and DEN-4 viruses were attenuated by passage in primary dog kidney cells (PDK). DEN-3 was attenuated by passage in primary green monkey kidney (PGMK) and final vaccine was produced in fetal rhesus lung cells (FRhL). The passage history of the four vaccine candidate is the following: DEN-1 (strain 16007) PDK-13; DEN-2 (strain 16681) PDK-53; DEN-3 (strain 16562) PGMK-30; FRhL-3 and DEN-4 (strain 1036) PDK-48. Phenotypic markers associated with potential attenuation of the four dengue candidate vaccines include temperature sensitivity, small plaque size in LLCMK2 cells, avirulence or increased survival time in suckling mice, and reduced or nondetectable viremia in monkeys (Yoksan *et al.*, 1986). Comparison of the nucleotide sequences of DEN1-4 parental strains and the vaccine strains derived therefrom confirms that the range of potential attenuating mutations in the flavivirus genome is

quite extensive (Tables I and II). No sequence comparisons are possible for the dengue 3 and 4 candidates because the parental strains have not been sequenced. In the case of DEN-1, the attenuated vaccine contains 5 amino acid mutations in the E gene and 3 mutations in nonstructural genes (Table I). In the case of dengue type 1, all but one of the mutations in the E gene occur in the hinge region between domains I and II. The hinge region is believed to play an important role in early events during infection of the cell. It undergoes a conformational change under low pH conditions, resulting in conversion of the E protein from a dimeric to trimeric form and outward projection of domain II bringing its tip into juxtaposition with the endosomal membrane. The result is fusion of the viral envelope and the endosomal membrane and subsequent virus release into the cytoplasm. Mutations in the hinge region may alter the ability of the E protein to undergo the required conformational alteration at acidic pH and lead to attenuation (Lee *et al.*, 1997). Interestingly, in the case of DEN2 PDK53 vaccine, the likely genetic determinants of attenuation are located in the nonstructural genes (Table II), making this virus strain attractive for the development of chimeric DEN virus as vaccine candidates (Huang *et al.*, 2000). Monovalent, bivalent, trivalent, and tetravalent vaccine formulations have been evaluated in adult volunteers in Thailand. A tetravalent dengue formulation has been evaluated in children in Thailand. Bhamarapravati and Yoksan (1997) have summarized the preclinical and early clinical studies. The vaccines appear to be immunogenic and well tolerated. A combined vaccine, in a tetravalent formulation, was the strategy selected for further investigations based on preliminary studies performed in Thailand (Bhamapravati and Yoksan, 1997). In 1993, the project was transferred to Aventis Pasteur in Lyon, France, to produce the dengue vaccines in a GMP facility at the industrial scale. Starting from the master seed produced in Thailand, working seed and vaccine lots for the four dengue serotypes were produced on the same cell substrate (PDK DEN 1,2,4, and FRhL DEN3) and at the same passage level. Intensive characterization has been performed to qualify these four viral strains in accordance with WHO requirements. Monovalent and tetravalent vaccines were lyophilized in a specific stabilizer containing chemically defined components.

Three double-blind, placebo-controlled studies have been performed with this vaccine (Table III). The major conclusions obtained during the evaluation of tetravalent live-attenuated dengue vaccine (Aventis Pasteur/Mahidol University) follow. Monovalent and tetravalent dengue vaccines caused clinical reactions in an acceptable range,

TABLE I
SUMMARY OF NUCLEOTIDE AND AMINO ACID DIFFERENCES BETWEEN THE GENOMES OF DEN-1 16007 VIRUS AND ITS VACCINE DERIVATIVE, STRAIN PDK-13

Nucleotide*	Gene-Amino Acid	16007	PDK-13	Amino Acid Change (16007 → PDK-13)	
1323	E-130	T	C	Vz	A
1541	E-203	G	A	Q	K
1543		A	G		
1545	E-204	G	A	R	K
1567	E-211	A	G	Silent (Q)	
1608	E-225	C	T	S	L
2363	E-477	A	G	M	V
2695	NS1-92	T	C	Silent (D)	
2782	NS1-121	C	T	Silent (A)	
5063	NS3-182	G	A	E	K
6048	NS3-510	A	T	Y	F
6806	NS4A-144	A	G	M	V
7330	NS4B-168	A	G	Silent (Q)	
9445	NS5-624	C	T	Silent (S)	

*1541 and 1543 were the first and third positions of a codon; mutations at both positions changed the codon for Q to K at E-203.
From Huang *et al.*, 2000.

consisting of mild to moderate and transient symptoms of fever, headache, myalgia, and rash. Vaccine formulations containing only 1 log of dengue 3 virus induced fewer reactions and this was selected for the trial in children. The clinical data have been reported in detail in previous papers (Kanesa-thasan *et al.*, 2001; Sabchaeroen *et al.*, 2002).

During the clinical studies, some of the volunteers were found to be seropositive for flavivirus infection (dengue, JEV, or both) at vaccination. The reactogenicity profile observed in these seropositive recipients was identical to that observed with flavivirus-naive volunteers (Sabcharoen *et al.*, 2002). The second dose of vaccine at 6 months was a challenging step in these studies: with respect to the antibody dependent enhancement hypothesis, as it may be speculated that the second dose of dengue vaccine given to people having partial immunity to the four dengue serotypes could result in enhanced virus replication with severe dengue fever or DHF. However, this did not occur. The frequency of adverse reactions in these individuals after the second dose was significantly reduced, especially in the children (Sabcharoen *et al.*,

TABLE II
SUMMARY OF NUCLEOTIDE AND AMINO ACID DIFFERENCES BETWEEN THE GENOMES OF DEN-2 16681 VIRUS AND ITS VACCINE DERIVATIVE, STRAIN PDK-53

Nucleotide	Gene-Amino Acid	16681	PDK-53	Amino Acid Change (16681 → PDK-53)	
57	5′ UTR	C	T		
524	PrM-29	A	T	D	V
2055	E-373	C	T	Silent (F)	
2579	NS1-53	G	A	G	D
4018	NS2A-181	C	T	L	F
5270	NS3-250	A	A/T*	E	V
5547	NS3-342	T	C	Silent (R)	
6599	NS4A-75	G	C	G	A
8571	NS5-334	C	T	Silent (V)	

*Two genetic populations were identified for this locus in the virus. The order of the two nucleotides reflects relative peak heights of the nucleotide signals in sequence chromatograms.

From Butrapet *et al.*, 1999.

2001, 2002) Immune responses to the vaccine following the first dose and the second dose of monovalent and tetravalent vaccines are summarized in Table IV. Monovalent live-attenuated dengue vaccines appear to induce type-specific antibody responses. The geometric mean titer (GMT) at 28 days after vaccination was 171, 661, 749, and 1092 for DEN1-4, respectively. The immunization with dengue tetravalent vaccine resulted in interference in antibody production to each of the four serotypes. DEN-3 virus was the predominant virus and was responsible for the interference (Kanesa-thasan *et al.*, 2001). This type of interference was also observed in the Thailand studies in both the adult and the children in whom the formulation was adjusted to contain only 1 log of dengue 3 virus and 2 or 3 logs of the other serotypes (Table IV). The magnitude of GMT is about 1–2 log lower in the tetravalent vaccine as compared to the monovalent vaccine except for DEN-3 (Kanesa-thasan *et al.*, 2001; Sabcharoen *et al.*, 2002).

In the Thailand adult study the percentage of volunteers who seroconverted to all four serotypes after the second dose was 70.7% (Sabcharoen *et al.*, 2002). One year after the vaccination, the percentage of volunteers who remained seroconverted to all the serotypes was 51%. Children age 5 to 12 years were immunized with a tetravalent dengue dose adjusted vaccine at 3- to 5-month intervals (Sabcharoen

TABLE III
CLINICAL PHASE 1 STUDY PERFORMED WITH AVENTIS PASTEUR/MAHIDOL LIVE-ATTENUATED DENGUE VACCINES

Place/Age	Number of Volunteers	Vaccine Formulation	Schedule and Virus Concentration
US adults	20	Monovalent	Five volunteers vaccinated with $4\log_{10}$ of a single dose of each dengue serotype.
US adults	10	Tetravalent	Single dose containing $4\log_{10}$ of each dengue serotype.
Thailand adults	49	Tetravalent	Seven formulations. Two doses at 6-mo interval.
Thailand children	82	Tetravalent	Two formulations with $1\log_{10}$ of DEN3 and 2 or $3\log_{10}$ of DEN1, 2, and 4. Two doses at 3–5 mo intervals.

et al., 2001). After the second dose, 61.3% seroconverted for all four serotypes (Table IV).

These results show that the two-dose tetravalent formulation strategy appears to be the best approach to induce immunity against the four dengue serotypes. The most obvious explanation is that the primary immunization provides full protection to the interfering serotype (dengue 3) and that the second dose is followed by a rapid neutralization of dengue 3, which therefore cannot interfere with replication of the other serotypes.

A total of 10 volunteers (six adults and four children) had neutralizing antibodies for dengue or JE viruses on the day of the first vaccination. All these volunteers developed a strong immune response against the 4 dengue serotypes and against JE virus. This typical secondary flavivirus response was associated with a ratio IgM/IgG <1.8 analyzed on day 16 and day 28. This ratio is a good marker to distinguish primary from secondary dengue infection (Vaughn *et al.*, 1997). Surprisingly, eight adults and six children who were thought to be negative for DEN 1-4 and JEV antibodies with hemagglutination inhibition (HAI), enzyme-linked immunosorbent assay (ELISA), and plaque reduction neutralization (PRNT) tests presented typical secondary dengue responses following the first immunization. A broad immune response was observed against DEN 1–4 and JEV, which was defined as a secondary infection having an IgM/IgG ratio <1.8 (Sabcharoen *et al.*, 2002). Similarly, during the US study in young

TABLE IV
IMMUNOGENICITY OF MONOVALENT AND TETRAVALENT DENGUE VACCINES IN VOLUNTEERS RECEIVING ONE AND TWO DOSES (AVENTIS/MAHIDOL VACCINES)

Vaccine and Study	Number of Volunteers	% Seroconversion (PRNT50)				% Seroconversion (PRNT50)	
		DEN1	DEN2	DEN3	DEN4	3 Dengue Serotypes	4 Dengue Serotypes
Monovalent US study	20	60	100	100	100	—	—
Tetravalent US study	10	40	20	100	10	10	0
Thailand adults							
1st dose	43*	76.7	60.4	100	39.5	58	34.8
2nd dose	41	85	78	100	70.7	75.6	70.7
Thailand children							
1st dose	78*	61.5	46.1	96.1	35.8	50	21.8
2nd dose	75	89.3	86.6	100	66.6	85.3	61.3

*Volunteers with DEN 1–4 or JEV neutralizing antibodies at the time of the inclusion were excluded from the analysis.

adults, three volunteers who were thought to be seronegative for YF, DEN 1–4, JEV, and SLE infections, also presented a typical secondary response following vaccination with dengue monovalent vaccines. It was established later that these volunteers had been previously vaccinated against YF 17D (Kanesa-thasan *et al.*, 2001). The immune response following the first dengue vaccination facilitated classification of the volunteers into three groups: a) naïve for flaviviruses with a high level of specific dengue IgM; b) volunteers immune to dengue or JEV; and c) volunteers whose antibody levels determined by conventional serologic tests (HAI, ELISA, PRNT) were negative but after vaccination the immune response was identical to a secondary dengue infection. Data from this last group suggest that these individuals were infected with a flavivirus not included in the screening (possible West Nile virus) or that at the dilution used (1:10) the PRNT was insensitive.

After the second dose of tetravalent dengue vaccine, a classical broad immune response against the four dengue viruses was expected (Innis *et al.*, 1997). In contrast, the vaccine induced a specific increase in antibodies for one or more of the dengue viruses with 60–70% of the volunteers having neutralizing antibodies against the four serotypes (Table IV).

There is a difference in the immune response between volunteers who had preexisting naturally acquired antibody against dengue or JE viruses and exhibited a broad immune response after receiving the first dose of tetravalent dengue vaccine, and volunteers naïve for flavivirus, who acquired partial immunity after the first dose of the tetravalent dengue vaccine, and exhibited responses only to some serotypes after the second dose. This phenomenon was observed in adults and in children after a 3- to 6-month interval between the first and second dose of vaccine (Sabcharoen *et al.*, 2001, 2002).

Kinetics of the dengue viremia have been determined only in adults (Sabcharoen *et al.*, 2002). Using different techniques (polymerase chain reaction, mosquito or cell virus isolation), viremia was detected after the first dose from day 2 to day 16. More than 95% of the volunteers developed a viremia for dengue 3 virus after the first dose. There is a clear inverse relationship between neutralizing antibody prevalence and viremia; as all the volunteers having neutralizing antibodies against DEN 3 after the first dose developed no viremia after the second dose. For the other serotypes, the highest prevalence of neutralizing antibodies (DEN1) is associated with the lower viremia, and reciprocally, a low induction of neutralizing antibodies for dengue 4 is associated with a predominant viremia.

2. WRAIR Tetravalent Lived-Attenuated Dengue Vaccine

Four wild dengue strains were attenuated after passage in PDK cells and the vaccine was produced in FRhL cells. The passage history of the four vaccine strains is the following: DEN-1 (strain 45AZ5) PDK20 FRhL3; DEN-2 (strain S16803) PDK 50 FRhL3; DEN-3 (strain CH53489) PDK 20 FRhL3; DEN-4 (strain 341750) PDK20 FRhL 4. Several phase 1 trials have been performed in the United States. The WRAIR DEN 2, 3, and 4 vaccine viruses were well tolerated. The DEN 1 vaccine was associated with a significant increase in reactogenicity. Inoculation of the tetravalent dengue virus vaccine was not associated with viral interference of antibody responses to each dengue virus. Following the second dose of vaccine a small booster effect was observed. From these preliminary data, intensive investigations were conducted to select the best vaccine formulation. Sixteen different tetravalent formulations were tested in groups of three to 10 volunteers. Seroconversion rates (for 16 formulations) were 83% to DEN1, 65% to DEN2, 57% to DEN3, and 25% to DEN4 after 1 dose and 94% to DEN1, 76% to DEN2, 70% DEN3 and 47% to DEN4 after two doses. To further explore the potential of the WRAIR tetravalent vaccine, two formulations (14 and 17) were evaluated in a large phase 1 trial. In these formulations, the PDK

passage levels differ for the DEN1 and DEN4 components. In formulation 17, the DEN1 PDK passage level was increased from 20 to 27 to further attenuate this component, and the DEN4 PDK passage level was decreased from 20 to 6 to reduce the attenuation of this component. Each formulation was administered as two doses (10^5–10^6 pfu), delivered at 6 months apart. Reactogenicity was considered mild to moderate. After two doses, tetravalent antibody seroconversion was achieved in 63% (formulation 17) or 32% (formulation 14) of vaccinees (WHO, 2002).

E. Conclusion

Development of live-attenuated DEN vaccines has a long history. The lack of a suitable animal model renders it difficult to select virus strains that maintain a balance between attenuation and immunogenicity. Classical tests such as temperature sensitivity, small plaque, and reduced neurovirulence in suckling mice, are not suitable markers of attenuation of dengue vaccine strains. However these *in vitro* and *in vivo* markers can be used as quality control tests to evaluate the consistency in vaccine batch production. The monkey animal model has played a major role in the development of live-attenuated candidate vaccines for dengue. Low viremia following vaccination appears to be a marker of attenuation and was used for the selection of attenuated dengue strains (Innis *et al.*, 1988). Monkeys have also been used to evaluate the immune response and play a major role in the choice of the strategy to use a combination of the 4-dengue virus serotypes in a single formulation. However, monkey vaccination cannot predict virus serotype interference observed in human trials with the dengue tetravalent vaccine (Kanesa-thasan *et al.*, 2001). Monovalent vaccines have been intensively used in human trials, but the data obtained do not predict the response to the tetravalent vaccine in humans. Experience acquired during the last three years reinforces the importance of working with the tetravalent vaccine approach (Kanesa-thasan *et al.*, 2001; Sabcharoen *et al.*, 2002). Adjustment of the different components of the vaccine to minimize interference has been a difficult step to overcome. Tetravalent live-attenuated dengue vaccine is the first injectable vaccine, which combines different serotypes of the same viral species. This could explain the interference observed and may be related to a competition in replication at the site of inoculation. Recent data from two tetravalent live-attenuated dengue vaccines showed that it is possible to obtain a good immune response with a two-dose schedule. Optimization of the formulation and the interval of time between the two doses remain major development issues.

There is no surrogate marker for immune protection against dengue disease. Neutralizing antibodies are thought to play a major role in protection, and evaluation of their role is currently being used as the reference test. Neutralizing antibody endpoints for protection need to be determined. The fact that volunteers with nondetectable neutralizing antibodies in conventional tests react after vaccination with an antibody response that resembles a secondary dengue infection, suggests that the PRNT test is not sensitive enough and, therefore, the detection of any neutralizing antibody can be considered as the endpoint for serologic testing. Analysis of immune responses after dengue infection is complicated by the induction of heterologous neutralizing antibodies whose role in protection or enhancement of disease is still unknown. The role of the anamnestic response in people previously exposed to flaviviruses infections is an important issue in defining a vaccination strategy. In hyperendemic countries like Thailand, more than 70% of the children older than 5 years of age have antibodies against flaviviruses. This percentage will increase with the inclusion of a JE vaccine in the Expanded Program of Immunization. As shown in the Thailand study involving a population with preexisting flavivirus immunity, a single injection of dengue live attenuated vaccine will induce a broad immune response against the four dengue serotypes. The value of this anamnestic response in terms of protection deserves to be further investigated.

Challenge of volunteers immunized with the tetravalent vaccine with wild type dengue strains to determine protection from clinical disease also need to be evaluated. Virus challenge could also be performed using a high concentration of monovalent dengue vaccine with the objective to determine a significant reduction in the viremia level that can represent a surrogate marker of disease protection. These investigations may be needed before the evaluation of the tetravalent live-attenuated dengue vaccine in an efficacy trial.

II. Japanese Encephalitis Virus Vaccine

Japanese encephalitis (JE) is the leading cause of childhood viral encephalitis in Asia. Every year almost 50,000 cases occur in this region, with a case-fatality rate of 5–35% (Tsai *et al.*, 1999). Inactivated JE vaccines have been used for many years and have led to control of the disease in Japan, Taiwan, and Korea. The Biken vaccine produced in Japan and elsewhere in Asia is the only one vaccine currently available internationally. The vaccine is licensed as JE-VAX in the United

States and distributed by Aventis Pasteur. The Nakayama JE strain used in the vaccine is produced by intracerebral inoculation of adult mice. The vaccine virus is purified and inactivated by formalin treatment. Two or three doses are given in the primary immunization series, followed by annual boosters to maintain immunity. Another inactivated JE vaccine is licensed only for use in China. The P3 strain of JE virus is used in this vaccine, which is produced in primary hamster kidney cells. The final vaccine is obtained from the crude culture harvest, inactivated and stabilized with albumin.

A live-attenuated JE vaccine for humans has been licensed in China since 1988. More than 30 million doses are annually produced in two institutions (Chengdu and Wuhan Production Institutes). The use of this vaccine has been described in detail by Tsai *et al*. (1999). Only a brief summary will be reported in this chapter in which we will discuss the position of the vaccine in terms of safety and international requirements.

A. Live-Attenuated Japanese Encephalitis SA14-14-2 Vaccine

The parental virus strain (SA14) was isolated from *Culex pipiens* larvae in 1954. The vaccine was obtained by the empirical process of serial passage, principally on primary hamster kidney (PHK) cells, plaque selection, and cloning in chick embryo cells. Subpassage in subcutaneous tissue of suckling mice followed by two cloning passages in PHK cells were necessary to obtain a stable non-neurovirulent virus (SA14-14-2). Compared to the JE parent strain that kills weanling mice by subcutaneous or intracerabral inoculation with an LD_{50} of $>10^{5.5}$, the attenuated strain is avirulent. The strain SA14-14-2 does not kill weanling hamsters inoculated intracerebrally. In addition, inoculation of rhesus monkeys by the intrathalamic and intraspinal routes does not produce clinical illness. Further evidence of the reduction in the neurotropism of the SA14-14-2 was obtained from experimental studies in athymic nude mice. No death or histopathologic alterations were observed after inoculation of 7 logs of virus in this animal model (Tsai *et al*., 1999).

Phenotypic characteristics of the SA14-14-2 strain, including small plaque size and reduced mouse neurovirulence, were stable after 10 passages in PHK cells beyond the vaccine passage level used for production. The vaccine is produced in PHK cells obtained from 10- to 12-day-old golden Syrian hamsters maintained in a closed colony at the Chengdu Biologics Institute. Monolayers are inoculated with a dilution of the working seed in a medium containing antibiotics and

albumin. After 3 to 4 days, the virus is harvested and filtered, and the resulting liquid vaccine is lyophilized in the presence of a stabilizer consisting of gelatin (1%) and sucrose (5%). The vaccine titer must exceed $10^{6.7}$ $TCID_{50}$ per milliliter. Potency testing is done in mice by measuring protection against the virulent P3 JE virus. The lyophilized vaccine is stable at 37 °C for one week, and for almost one year at 4–8 °C. The vaccine is given annually, during spring campaigns, to infants older than 1 year of age. Since 1988, the SA14-14-2 has been administered to over 100 million Chinese children without apparent complications. Clinical monitoring of experimentally immunized subjects documented the absence of local or systemic symptoms after immunization. The vaccine safety was established among 1026 children who were followed for 14 days after a single dose of the vaccine. No cases of encephalitis or other severe events were reported (Xin *et al.*, 1988). Short-term safety (30 days) was demonstrated in a large randomized trial involving 26,239 subjects, in which the incidence of adverse events in the vaccinated group was no higher than that of the control group (Lui *et al.*, 1997).

Pre-licensure data in prospective non-randomized trials conducted in highly endemic areas indicated 95% vaccine efficacy after a single dose (Tsai *et al.*, 1999). A recent case-control study in less endemic countries showed 80% effectiveness after one dose and 97.5% after two doses administered 1 year apart (Hennessy *et al.*, 1996).

The first clinical evaluation of the SA14-14-2 vaccine outside China was performed in 84 children between 1 and 3 years of age in Korea. The vaccine was safe, with no significant adverse events noted. After a single dose, 96% of the children seroconverted. Some of the children had previous immunity against JE that was acquired either by vaccination with JE-inactivated vaccine or by natural infection. In this group, the vaccine provides a booster effect manifested by a high level of neutralizing antibodies (Sohn *et al.*, 1999).

Only limited data are available on the immune status before vaccination. The lower effectiveness (80%) of a single dose of SA14-14-2 in the study performed by Hennessy *et al.* (1996), as compared to the 95% efficacy rate in the prospective non-randomized trial, has been attributed to the level of the transmission of the disease in different areas. In highly endemic JE areas, the immunity provided by a vaccination could be reinforced by previous or subsequent natural exposure to JE virus. This hypothesis is supported by previous immunogenicity studies showing that the two-dose regimen of inactivated JE vaccine is adequate in an endemic area, but three doses are needed to stimulate protective immunity in non-endemic areas (Tsai *et al.*, 1999).

Interestingly, in the Korean study of 68 children vaccinated with SA14-14-2, only nine (13%) children developed virus specific IgM antibodies after primary immunization (Sohn *et al.*, 1999). It is possible that the absence of specific IgM reflect a previous exposure to a flavivirus, although the study was performed in an area of low JEV endemicity.

B. Genetic Basis of Japanese Encephalitis Virus Attenuation

The genome sequence has been determined for the parental SA14 virus (from several different laboratory passages) and the attenuated vaccine, SA14-14-2, grown in primary hamster kidney cells (PHK) (Aihara *et al.*, 1991) and primary dog kidney cells (PDK) (Nitayaphan *et al.*, 1990). Six common amino acid differences in the E gene at positions E107, E138, E176, E279, E315, and E439 distinguished the SA14-14-2 vaccine from parental virus (Table V). Ni *et al.* (1994) compared four attenuated vaccine strains: SA14-14-2 PHK, SA14-14-2

TABLE V
COMPARISON OF THE AMINO ACID DIFFERENCES IN THE E PROTEIN OF JE SA14-14-2 VACCINE AND THE WILD-TYPE SA14 PARENT OF THE VACCINE STRAIN* (FROM NI *ET AL.*, 1994)

Amino Acid	SA14 Parent†	SA14-14-2 PHK‡	SA14-14-2 PDK§	SA14-5-3¶	SA14-2-8¶
E107	**Leu**	**Phe**	**Phe**	**Phe**	**Leu**
E126	Ile	Ile	Ile	Ile	Thr
E128	Arg	Arg	Arg	Arg	Lys
E138	**Glu**	**Lys**	**Lys**	**Lys**	**Lys**
E176	**Ile**	**Val**	**Val**	**Val**	**Val**
E177	Thr	Ala	Thr	Thr	Thr
E179	Lys	Lys	Lys	Lys	Glu
E243	Glu	Glu	Lys	Glu	Glu
E244	Glu	Gly	Gly	Gly	Gly
E264	Gln	His	Gln	Gln	Gln
E279	**Lys**	**Met**	**Met**	**Met**	**Lys**
E315	**Ala**	**Val**	**Val**	**Val**	**Val**
E439	**Lys**	**Arg**	**Arg**	**Arg**	**Arg**

*From Ni *et al.*, 1994.
† SA14 (USA), Ni *et al.*, 1994.
‡ Aihara *et al.*, 1991.
§ Nitayaphan *et al.*, 1990.
¶ Ni *et al.*, 1994.
The differences in **bold** are considered important to the full attenuation phenotype of the SA14-14-2 vaccine.

PDK, and the precursor vaccine candidates SA14-2-8 and SA14-5-3, with SA14 viruses, and in a later paper, Ni *et al*. (1995) revised this analysis based on a corrected sequence for one SA14 substrain. These studies demonstrated that four common amino acid changes at positions E138, E176, E315, and E439 separated the various vaccine and parental wild-type viruses. The authors suggested a role for these mutations in virulence. The importance of the E138 site was further emphasized by Sumiyoshi *et al*. (1995), who showed that substitution of a basic amino acid for glutamic acid at this residue changed a recombinant virulent JE virus to an attenuated phenotype. Because the SA14-14-2 vaccine virus has been used to construct a chimeric yellow fever vaccine (Chambers *et al*., 1999; see Lai and Monath, this volume), efforts have been made to define the attenuating mutations in the prM-E structural genes used as donors in the chimerization. This analysis used a series of intratypic yellow fever/JE chimeric viruses containing either single or multiple changes in these E gene amino acid residues. The role of single and multiple revertants in restoring neurovirulence to the chimeras was studied. Different combinations of reversions restored a neurovirulence phenotype, but residue E138 exhibited a dominant effect. By studying the effects of various mutation clusters, residues E107, E176/177, and E279 also were implicated in neurovirulence (Arroyo *et al*., 2001).

C. Production and Regulatory Issues

The JE SA14-14-2 vaccine is produced in PHK cells. Primary cell cultures of different sources have been used worldwide for production of live vaccines for human use for more than 30 years. The production and quality control of the cells and the vaccine are submitted to specific requirements (WHO, 1987). The research on adventitious agents is performed on control cells and bulk vaccine after neutralization of the virus. The tests are limited to the detection of haemabsorbing viruses and viruses that cause detectable cytopathic effect after inoculation in three different cells lines, one of which is a human diploid cell line. These tests, developed in the 1960s, have facilitated the detection of a limit number of adventitious agents. Virus-like HBV, HIV1, HIV2, HTLV1, and HCV, which are of major concern in the transmission of infectious agent from biologic products, cannot be detected by the tests commonly used for the detection of adventitious agents.

In the past, live-attenuated vaccines have been a source of transmission of infectious agents. The yellow fever 17D vaccine was responsible for a dramatic outbreak of jaundice in 1942, with approximately 28,000

cases and 62 deaths from fulminant hepatitis (Sawyer *et al.*, 1944) due to the contamination of the vaccine by a hepatitis B virus present in the human serum used as stabilizer (Seeff *et al.*, 1987). The YF17D vaccine has also been the origin of the transmission to humans of avian leucosis virus. The YF17D vaccine produced in different facilities was contaminated with 5–6 $\log_{10}$/mL of avian-leucosis virus. The contaminating virus did not interfere with the replication of YF17D and was not detectable by tests currently used for the research of adventitious agents. A retrospective survey of those vaccinated with this contaminated vaccine did not show an increased risk of leukemia or other malignancies (Melnick, 1968). Similarly poliovirus vaccines produced in primary monkey cells transmitted SV40 virus to humans during the vaccination campaigns in the early 1960s. The long-term consequences of this exposure are still under evaluation.

The Chinese SA14-14-2 vaccine presents some concerns related to the origin of the primary cells and the technique used for their production that may increase the risk for transmission of adventitious agents. There is no precedence for the use of PHK cells for the production of live-attenuated vaccines. Hamster cells are known to harbor retroviruses and there is no specific requirement for testing this group of viruses. Generally primary cells are prepared from tissues obtained from specific pathogen-free (SPF) animals. This requires careful screening of the animals for the absence of antibodies to specific viruses which limits the risk of contamination. The production of SA14-14-2 virus in primary cells are not from SPF animals but obtained from a closed colony. Furthermore, the productivity of this vaccine is low, as a batch of one million doses requires 330–500 hamsters (Tsai, 2000). Every year, approximately 10,000 animals would be needed to produce this vaccine. Another production issue for the SA14-14-2 vaccine is the direct harvest from supernatants of infected cells without any purification step. Raw materials used for the production of the SA14-14-2 vaccine (bovine calf serum, trypsin, human albumin and gelatin) can be also a source of transmission of contaminating agents.

The quality control of this vaccine must be improved, particularly the inclusion of very sensitive tests such as PERT assay, for the detection of retroviruses. Alternatively, modern technology can be applied to improve the production of the vaccine. For example, the production of a well-characterized PHK cell bank and the use of cells further amplified in culture is a potential way to decrease the number of hamsters used while lowering the risk of adventitious virus transmission. Another efficient way may be the adaptation of the virus to suitable continuous cell lines. This can be achieved after virus RNA extraction followed

by transfection of the approved cells. The change of cell substrate is a major issue for production of live-attenuated vaccines in terms of safety and immunogenicity. SA14-14-2 virus has been well characterized, the full genome sequence has been published, the major determinant of attenuation for mice is established, and phenotypic markers of pathogenicity for animals are well documented. The rationale for development of a modern vaccine for safety reasons appears realistic. Immunogenicity of the derived vaccine will be a major concern. Nevertheless, taking into consideration that the immunogenicity of the SA14-14-2 vaccine in humans is dose-dependent (Xin *et al.*, 1988), a dose-range study needs to be done to optimize the formulation. The need to increase the virus concentration in the final vaccine should not be a major issue in terms of productivity. Virus yields are expected to be higher particularly from Vero cells expanded in microcarrier technology. Whenever possible, the substitution of raw material from human or animal origin have to be part of the development of the new vaccine.

Very little doubt exists on the safety and efficacy of the SA14-14-2 vaccine, but improvement on the quality of this vaccine is needed for an international acceptance.

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ADVANCES IN VIRUS RESEARCH, VOL 61

DNA VACCINES FOR FLAVIVIRUSES

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I. Introduction

DNA vaccines offer potential solutions to some of the obstacles encountered in developing vaccines for flaviviruses. For example, vaccine interference due to preexisting antibodies to other flaviviruses or to a vaccine vector is not a problem with DNA vaccines. In addition, it should be possible to readily combine DNAs encoding the antigens of a number of flaviviruses to produce multiagent vaccines. Attempts to develop DNA vaccines for flaviviruses have been ongoing for several years, aided by advances in our understanding of the molecular biology of these viruses. Key developments include the molecular cloning and sequence analysis of several medically important flaviviruses beginning with yellow fever virus (Rice *et al.*, 1985); the elucidation of the crystal structure of the envelope (E) glycoprotein of tick-borne encephalitis virus (Heinz *et al.*, 1991); and the many studies of flavivirus gene expression, protein processing and secretion (reviewed in Lindenbach and Rice, 2001).

The E gene is the principal candidate for use in flavivirus DNA vaccines. It encodes the major surface antigen of the virion, which contains immunologically important epitopes (reviewed in Lindenbach and Rice, 2001). Antibodies directed against epitopes on E neutralize

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the viruses and are important mediators of protective immunity (reviewed in Burke and Monath, 2001). Work with vaccinia virus and other mammalian cell expression systems further showed that interaction of E with another structural protein, prM, is essential for intracellular processing and secretion of E in the correct conformation (reviewed in Burke and Monath, 2001). Consequently, for most DNA vaccine studies, the prM and the E genes have been coexpressed. A few studies have evaluated candidate DNA vaccines encoding nonstructural proteins (NS1 or NS3), because of earlier work demonstrating that these proteins also elicit protective immune responses in animal models (Chen *et al.*, 1999; Lin *et al.*, 1998; Morozova *et al.*, 2000; Schlesinger *et al.*, 1987). In general, studies to date indicate that optimal coexpression of the complete prM and E genes results in the best immunogenicity for flavivirus DNA vaccines.

In addition to considering which flavivirus genes to express, development of effective DNA vaccines requires the consideration of gene promoter and enhancer elements for optimal expression, the vaccine delivery device and the route of inoculation for efficient targeting of antigen processing in cells, and the possible need for accessory factors (i.e., genetic adjuvants), such as cytokine genes or immunostimulatory sequences. Most flavivirus DNA vaccine efforts have been performed using plasmid vectors with a cytomegalovirus (CMV) immediate early promoter to control transcription, although Rous sarcoma virus (RSV) (Chang *et al.*, 2000) and SV40 (Allison *et al.*, 1995) promoters have also been used.

As in many other DNA vaccine studies, the vectors used most frequently in flavivirus DNA vaccine experiments have nucleotides encoding bovine growth hormone polyadenylation signals for transcription termination. For selection in bacteria, either ampicillin or kanamycin resistance genes are included. The ampicillin resistance gene contains more immunostimulatory CpG motifs for mice than does the kanamycin resistance gene (Sato *et al.*, 1996), which might bias some of the studies conducted in mice. The kanamycin resistance gene is more suitable for vaccines destined for clinical studies, because of concerns that the ampicillin gene might be incorporated into pathogenic bacteria when inoculated into humans, thus rendering them resistant to common antibiotic treatment. In contrast, kanamycin and other aminoglycoside antibiotics such as neomycin are not extensively used to treat clinical infections so development of resistant bacteria would be less problematic (FDA, 1996).

A variety of DNA vaccine approaches have been studied in animals, many with promising results. Clinical studies to support the potential of

DNA vaccines are currently lacking. In this review we will summarize efforts to develop DNA vaccines for a number of flaviviruses and studies to evaluate them in animal models.

II. DNA Vaccines for Dengue Fever

The first reported studies with DNA vaccines for dengue (DEN) virus involved expression of the DEN-2 prM gene and the N-terminal 92% of the E gene from two different CMV promoter-containing expression vectors (Kochel *et al.*, 1997). Transfecting cells in culture demonstrated correct E gene expression from the DNA. Dengue polyclonal and monoclonal antibodies reacted with the expression products as measured by immunofluorescent antibody assays (IFA) and immunoprecipitated the E protein from the supernatant fluid. To assess the immunogenicity, BALB/c mice (10 per group) were injected intradermally (i.d.) with 200 μg of each DNA on days 0, 9, 22, and 57. Most mice had antibodies measured with enzyme-linked immunosorbent assay (ELISA) after the second inoculation and all demonstrated anti–DEN-2 neutralizing antibodies measured by a plaque reduction-neutralization test (PRNT) 35 days after the fourth inoculation, with 50% neutralizing antibody ($PRNT_{50}$) titers ranging from 1:10 to 1:320. Because mice become naturally resistant to lethal intracerebral (i.c.) challenge with DEN virus at approximately 5 to 6 weeks after birth, they have limited usefulness in vaccine protection studies. Nevertheless, the investigators administered a lethal DEN-2 virus challenge to 6-week-old mice that had received two doses of the DNA expressing prM-92%E beginning at 3 weeks of age. Perhaps not unexpectedly, no protection was observed. As the authors point out, this result was most likely due to the absence of neutralizing antibodies at the time of challenge.

A follow-up study by the same investigators examined the effect of co-delivery of the prM-92%E DNA vaccine with pUC9 plasmid DNA, which was included to provide additional immunostimulatory CpG motifs (for mice), which are found in the ampicillin resistance gene of the plasmid (Porter *et al.*, 1998). Administering two or three low (3.1 μg) doses of the prM-92%E DEN-2 DNA vaccine combined with 100-μg doses of pUC19 resulted in higher antibody titers as measured with ELISA and PRNT than were achieved with the DNA vaccine alone. To assess the protective efficacy of the vaccine plus pUC19 combination, groups of 3 week-old mice were given 12.5 μg of vaccine or 12.5 μg of the plasmid vector combined with 100 μg of pUC19. After inoculation on days 0 and 10, the mice were challenged with a lethal dose

of DEN-2 virus on day 21. Although anti-DEN-2 antibodies could not be measured with ELISA at the time of challenge, a statistically significant level of protection was observed in the vaccinated group, which showed 60% survival, compared with a non-vaccinated control group, which showed only 10% survival. The authors conclude that the generally positive results obtained in the mouse model warrant further study, including experiments in non-human primates.

In an attempt to improve the efficacy of the DEN-2 DNA vaccine, studies were performed aimed at targeting the expression products through the MHC class II pathway by adding the transmembrane and cytoplasmic portions of the lysosome-associated membrane protein (LAMP) to the construct expressing prM and 100% of E (DEN-2 ME100) (Raviprakash *et al.*, 2001). Expression studies showed the expected result of targeting of E protein to the lysosomes where it colocalized with endogenous LAMP. To test the immunogenicity of the constructs, groups of mice (n = 5) were inoculated i.d. on days 1, 11, and 21. The LAMP-containing construct elicited a more rapid and a higher rate of seroconversion (five of five vs. two of five), and a higher neutralizing antibody response (1:270 versus <1:10 at day 120) than the DEN-2 ME100 construct. Further studies, in which a plasmid encoding the mouse cytokine granulocyte macrophage–colony-stimulating factor (GM-CSF) was delivered with the DEN-2 DNA vaccine, resulted in a seroconversion rate of 100% and a fivefold to tenfold increase in neutralizing antibody titers, a significant improvement over that seen when the vaccines were administered alone (Raviprakash *et al.*, 2001). The authors suggest that the inclusion of LAMP allowed the E antigen to be more efficiently presented to CD4+ helper T-cells, thus resulting in increased antibody responses. The mechanism responsible for the increased antibody titers observed in the presence of GM-CSF may be different, perhaps a generalized increase in B- and T-cell helper activity. Overall, these studies suggest that the use of accessory factors may augment the efficacy of DNA vaccines for DEN viruses.

DNA vaccine candidates similar to those examined for DEN-2 virus were evaluated for DEN-1 virus. Plasmids were constructed containing prM and 92%E (D1ME92), prM with 80% of the E gene (D1ME80), prM with 100% of the E gene (D1ME100), and the N-terminal 80% of the E gene without prM (D1E80) (Raviprakash *et al.*, 2000a). Measuring expression in 293 cells transfected with the various constructs revealed that D1ME100, D1ME92, and D1E80 all produced products of the expected sizes, and these proteins were secreted from the cells. However, the D1ME80 expression product appeared to be degraded. The D1ME100 construct was further shown to produce extracellular,

virus-like particles (VLP) similar to natural viral slowly sedimenting hemagglutinin (SHA) particles (reviewed in Lindenbach and Rice, 2001). Immunogenicity of the DNAs was tested in BALB/c mice, which were inoculated i.d. with 100 μg of each plasmid on days 0, 11, 21, and 51. The best results were obtained with D1E80 and D1ME100, for which $PRNT_{50}$ titers were observed ranging from 1:10 to 1:40, and which persisted for longer than 6 months after primary vaccination.

Due to its immunogenicity and its ability to make secreted VLPs, the DEN-1 ME100 vaccine was further evaluated in rhesus macaques (Raviprakash *et al.*, 2000b). The animals were inoculated intramuscularly (i.m.) or i.d. with three or four doses of 1 mg of the DNA. The animals vaccinated i.m. appeared to seroconvert more rapidly and developed higher and more persistent antibody titers than animals vaccinated i.d. In contrast to results in mice (Raviprakash *et al.*, 2000a), co-administration of pUC 19 DNA appeared to have no significant effect on the immunogenicity of the vaccine in rhesus monkeys, regardless of the route of administration used. This result is likely due to the use of immunostimulatory sequences optimal for mice, rather than those known to be optimal for primates. At the time of the studies, the species specificity of immunostimulatory sequences was not known.

To assess protective immunity elicited by the DEN-1 ME100 DNA, the monkeys vaccinated i.m. were challenged with DEN-1 virus at month 9 and animals vaccinated i.d. were challenged at month 15 of the vaccination regimen. Although the rhesus monkey is not an authentic disease model for DEN, peripheral challenge of DEN-naïve animals results in a viremia that can be easily measured. In the DNA vaccine challenge studies, four of eight animals vaccinated i.m. had no detectable viremia after challenge. The other four vaccinated animals were viremic for 2–3 days, a duration slightly less than observed in the non-vaccinated controls. In contrast, no reduction in viremia after challenge was observed in those animals vaccinated i.d. The four animals that were completely protected against viremia also demonstrated the highest neutralizing antibody titers immediately after vaccination, although the titers declined, and most were undetectable by PRNT by the time that the viral challenge was performed. No correlation between the level of protection against viremia and antibody avidity was apparent (Raviprakash *et al.*, 2000b).

Measuring the anamnestic antibody responses of the vaccinated and challenged monkeys demonstrated both IgG and IgM responses beginning on day 7 after challenge and peaking on day 14 (regardless of the route of vaccination), while the non-vaccinated controls developed primary IgM-type responses (Raviprakash *et al.*, 2000b). DEN-1–specific

T-cell responses were assessed in two animals that were protected against viremia. Surprisingly, no interferon-γ (IFN-γ) production was observed in peripheral blood lymphocyte (PBL) cultures obtained after vaccination but before viral challenge and stimulated *in vitro* with live or inactivated DEN-1 virus. However, the same cultures stimulated with concanavalin A were positive for IFN-γ, and cultures obtained after challenge were also positive for IFN-γ. The authors argued that the number of T-cell epitopes on the vaccine may be small and that most of the T-cells may be in the lymphoid tissues rather than in the peripheral blood.

The DEN-1 ME100 DNA vaccine was further evaluated in a different non-human primate model, *Aotus nancymae* (Kochel *et al.*, 2000). The monkeys were given 1 mg of DNA plus 0.5 mg of pUC 19 i.m. (n = 3) or i.d. (n = 3). Controls received vector alone plus pUC 19 or no DNA. Vaccinated animals were boosted at months 1 and 5. All three animals inoculated by the i.m. route seroconverted by 2 months after the first booster, with neutralizing antibody ($PRNT_{50}$) titers of 1:40 to 1:80. These titers increased at least fourfold by 1 month after the second booster. At this time, two of three animals vaccinated by the i.d. route had also seroconverted with $PRNT_{50}$ antibody titers of 1:40 and 1:160. There was only a modest decline in the antibody titers up to the time the animals were challenged at month 11, and none of the seropositive animals became seronegative. After viral challenge at month 11, only two animals were completely protected against viremia; these two animals also had the highest neutralizing antibody titers immediately before challenge. Two other animals, one that was vaccinated i.m. and one that was vaccinated i.d., were partially protected with only 1 day of viremia. Viremia in the controls ranged from 2 to 5 days. An anamnestic antibody response was observed after challenge in the vaccinated animals, probably indicating replication of the challenge virus. Whether this type of immune response (i.e., suggesting partial immunity) would protect, or conversely might enhance DEN disease in humans should be considered. Also, in this series of experiments in *Aotus* monkeys, no clear advantage of i.m. delivery over i.d. delivery was observed, in contrast to previous work in other animal models.

Another group also evaluated a candidate DNA vaccine for DEN-2, which was constructed by cloning the full-length prM and E genes into a commercially available plasmid (pcDNA3, Invitrogen), which contains a CMV promoter and ampicillin resistance gene (Konishi *et al.*, 2000a). After verifying correct expression in CHO cells, these investigators vaccinated BALB/c mice by the i.m. route using different regimens. The lowest doses of DNA tested, 1 μg and 10 μg, failed to elicit

measurable anti-DEN-2 neutralizing antibodies after two inoculations. However, after two to three inoculations with 100 μg of the vaccine, $PRNT_{90}$ titers of approximately 1:10 were observed. When the vaccinated animals were subjected to a non-lethal, peripheral challenge with 5.5 $\log_{10}$ of live DEN-2 virus, they demonstrated a brisk anamnestic neutralizing antibody response to the challenge virus that was detected by days 4 and 8, in contrast to naïve controls, which responded much more slowly. Although this represents a failure of sterile immunity, the authors cite their results with Japanese encephalitis virus (JEV) vaccines where similar immune responses conferred protection against lethal, peripheral, JEV challenge (Konishi *et al.*, 1999) and other experiments that show protection after passive transfer of neutralizing antibodies (Kaufman *et al.*, 1987, 1989). They argue that such an antibody response indicates the presence of virus-responsive memory B-cells and suggests a state of protective immunity.

Another approach to DNA vaccines for DEN-2 virus involves delivery to skin by a gene gun. To prepare DNA for gene-gun delivery, the plasmid is precipitated onto the surfaces of micrometer-size gold beads. The DNA-gold is suspended in ethanol and dried under nitrogen on the inside surfaces of plastic tubing. The tubing is then cut into half-inch sections, and these cartridges are loaded into the chambers of a gun. The DNA-gold is discharged from the gun under helium pressure, which is adjusted so that most of the gold reaches the epidermis of the test animal. Recent studies indicate that almost all of the expression that results from gene-gun delivery involves cells that have at least one gold bead deposited directly into the nucleus. Apparently, very inefficient transfer of DNA from the cytoplasm to the nucleus, where transcription must occur, takes place. Compared with needle and syringe, gene-gun delivery more effectively targets the DNA to antigen-presenting cells, such as Langerhans cells, which are abundant in the skin. Consequently, far less DNA is required to elicit immune responses.

In one DNA/gene-gun study (Putnak *et al.*, manuscript in preparation), outbred Swiss mice vaccinated with 0.5 μg of DNA in a plasmid containing a CMV promoter and kanamycin resistance gene and the full-length DEN-2 prM and E genes, developed anti-DEN-2 neutralizing antibodies after a single inoculation, with $PRNT_{50}$ titers ranging from 1:50 to 1:380 (GMT 1:180). To assess cytotoxic lymphocyte (CTL) responses, BALB/c mice were vaccinated by gene gun with 0.5 μg of the DNA, boosted at 4 weeks, and spleen cells from the vaccinated mice were cultured with target cells expressing prM and E at various effector to target (E:T) cell ratios. Lysis of target cells increased

from fivefold to eightfold above the control as the E:T ratio was increased from 16:1 to 50:1. Several vaccination regimens were evaluated in rhesus monkeys. The results showed that two 2-μg doses effected a 100% rate of seroconversion, with neutralizing antibody ($PRNT_{50}$) titers ranging from 1:55 to 1:80. However, a single 2-μg dose was not effective at eliciting measurable neutralizing antibodies, demonstrating the need for a booster vaccination. Interestingly, however, the antibody titers achieved after the second dose did not increase significantly after a third or a fourth dose. DEN-2 virus challenge 1 month after the fourth dose showed complete protection against viremia in two of three vaccinated animals with only 1 day of viremia in the remaining animal, compared with 4, 4, and 6 days of viremia in the three naïve control animals. Reducing the dose of DNA to 1 μg resulted in lower rates of seroconversion. Thus, of nine animals that received a single 1-μg dose of DNA, only one seroconverted ($PRNT_{50} = 1{:}24$), and only two of six animals seroconverted after two 1-μg doses of DNA ($PRNT_{50} = 1{:}40$ and 1:45). Nevertheless, two of the three animals that received two 1-μg doses of DNA were completely protected against viremia and the third animal had only 1 day of viremia after challenge with DEN-2 virus 1 month after the second inoculation. In comparison, the controls in this experiment had 2, 3, and 5 days of viremia. Long-term protection with the vaccine could not be demonstrated; no protection against viremia was seen when animals that received two 1-μg doses of DNA were challenged 7 months after the second dose. In addition, elimination of the booster also had a negative effect on protection; no protection against viremia was seen in three animals that were challenged 1 month after a single 1-μg dose of DNA. In these experiments, although there was a positive correlation between the development of neutralizing antibodies and protection, some animals that failed to make neutralizing antibodies were also protected. However, none of the animals that were protected against viremia demonstrated sterile immunity, in that postchallenge neutralizing antibody titers of vaccinated animals were not significantly different from those of naïve controls. These results, while supporting the contention that neutralizing antibodies are important, may indicate a role for other factors, such as cell-mediated immune responses (CMI), in protection mediated by DEN DNA vaccines.

DNA vaccine for DEN-2 virus has also been tested in the context of a prime-boost regimen using recombinant envelope protein (amino acids 298–400 [B domain]) of DEN-2 expressed as a fusion protein with maltose binding protein (Simmons *et al.*, 2001). The combination of DNA and protein generated levels of neutralizing antibodies higher

than those elicited by DNA alone, and shifted the immunoglobulin subclass towards lgG1 production, in contrast to DNA and live virus, which biased toward lgG2a production. These results indicate that immune responses to DNA vaccines can be modulated by the prime-boost approach, which may be relevant to future strategies for optimizing use of this vaccine modality.

III. DNA Vaccines for Japanese Encephalitis

Several groups have evaluated candidate DNA vaccines for Japanese encephalitis virus (JEV) in mice (Ashok and Rangarajan, 2000; Chang *et al.*, 2000; Chen *et al.*, 1999; Kaur *et al.*, 2002; Konishi *et al.*, 1998, 1999; Lin *et al.*, 1998) and two groups reported studies using swine (Konishi *et al.*, 2000b; Nam *et al.*, 2002).

To evaluate the ability of JEV structural and nonstructural proteins to elicit protective immunity in mice, one group of investigators prepared pcDNA3 constructs expressing the C, E, NS1-2A, NS3 or NS5 genes of JEV (Chen *et al.*, 1999). They vaccinated groups of C3H/HeN mice three times at 3-week intervals by i.m. injection of 100 μg of DNA, or by gene-gun inoculation of 1 μg of DNA. Only the E gene constructs elicited substantial protection with 25 of 28 mice given i.m. injections and 21 of 23 given gene-gun inoculations surviving challenge. Despite protection, JEV-neutralizing antibodies could not be detected at the time of challenge, and high levels of neutralizing antibodies appeared after challenge. This result was not unexpected in that expression of E in the absence of prM generates a protein that has a non-native conformation. The authors concluded that the protection that they observed was due to the priming of the DNA vaccine and the subsequent memory B-cell response. The importance of the humoral response to this JE DNA vaccine for protection from JEV challenge was supported by the results of adoptive transfer experiments in which intravenous transfer of E-specific antibodies, but not crude or T-cell–enriched splenocytes to sublethally irradiated mice, conferred protection (Pan *et al.*, 2001).

In the same study, the investigators further examined the antibody isotypes elicited by the vaccine as a measure of T-cell helper activity. Consistent with earlier mouse studies (reviewed in Haynes, 1999), mice given the DNA vaccine by i.m. injection developed predominantly lgG2a antibodies, suggesting a T-cell-helper 1 (Th-1) type response, whereas mice given the DNA by gene gun had a higher lgG1/lgG2a

antibody ratio, suggesting a Th-2 type response. In a subsequent study, these investigators attempted to influence the magnitude of the helper response by coadministering (i.m. injection) their E-expressing construct with plasmids expressing IL-4 (to enhance the Th-2 response) or IL-12 (to enhance the Th-1 response) (Chen *et al.*, 2001). All 10 mice that received the E construct but neither of the cytokine genes survived challenge with JEV, whereas 8 of 10 mice receiving IL-4 and 3 of 10 receiving IL-12 along with the E construct survived. The investigators found that the mice that were given IL-12 were immunosuppressed with barely detectable antibody titers to E and reduced CTL responses.

Although the NS1 construct in the aforementioned study failed to provide protective immunity to mice, in a different study comparing plasmids expressing prM and E, NS1 only, or NS1 plus 60 amino acids of the amino terminus of NS2A, partial protection of ICR mice was elicited by both the construct expressing prM and E (seven of 10 survived) and the construct expressing NS1 (nine of 10 survived) (Lin *et al.*, 1998). In contrast, the NS1-NS2a construct and the plasmid without an insert protected four of 10 mice. The authors found that when NS1 was expressed alone, but not when expressed in combination with the amino terminal portion of NS2A, it was secreted and also found on the cell surface. The cell-surface expression is probably the reason for the protection observed, as evidenced by the ability of sera from the DNA-vaccinated mice to lyse JEV-infected cells in an antibody-dependent complement-mediated cytotoxicity assay (Lin *et al.*, 1998).

In studies by another group, a candidate DNA vaccine for JEV was constructed by inserting the prM and E genes of JEV, strain Nakayama, into pcDNA3 (Konishi *et al.*, 1998). Coexpression of the E and prM genes was expected to produce antigens with native conformation, able to elicit neutralizing antibodies. However, groups of 5 ICR mice, inoculated i.d. or i.m. with 10 μg or 100 μg of the construct (pcDNA3JEME) at 4 weeks and 6 weeks of age, were found to have no detectable neutralizing antibodies after the first vaccination, and only very low levels of neutralizing antibodies ($PRNT_{90}$ titers 1:10–1:20) 2 weeks after the second vaccination (week 8). Despite the unexplained absence of neutralizing antibodies after vaccination, upon challenge with 10,000 LD_{50} of the P3 strain of JEV at week 8, mice developed high levels of neutralizing antibodies ($PRNT_{90}$ 1:160–1:640), and all survived. The construct was also evaluated in ICR mice given only one i.m. inoculation at 4 weeks of age with 0.1–100 μg of DNA. No neutralizing antibodies could be detected in any of the mice at 3 weeks after

vaccination, and upon challenge at week 7, only the mice that had received the 100-μg dose developed neutralizing antibodies, and showed partial protection (four of five mice survived). To evaluate the duration of protective immunity elicited by pcDNA3JEME, groups of two BALB/c mice, 6 weeks old, were given one i.m. inoculation of 100 μg of the DNA then challenged at 1, 2, 4, or 6 months after vaccination. Although $PRNT_{90}$ titers were $\leq$1:10 at the time of challenge, all mice survived. As in the later study by Chen *et al*. (1999), the investigators concluded that the large increases in neutralizing antibody titers that arose after challenge indicate that the mice maintained sufficient memory B cells to produce high levels of protective neutralizing antibodies if challenged within 6 months of vaccination (Konishi *et al*., 1998).

In addition to neutralizing antibodies, the ability of the pcDNA3-JEME construct to elicit CTL responses was evaluated by vaccinating BALB/c mice once or twice with the DNA, harvesting spleen cells and restimulating them *in vitro* by infection with JEV, then measuring their ability to lyse MHC-matched target cells infected with JEV. To further characterize the CTL response, additional experiments were performed with target cells infected with recombinant vaccinia viruses expressing prM and E, or prM, E, NS1, or E and NS1. In all experiments, cytotoxic activity was detected. The results were interpreted as indicating that specific CTL responses, primarily to the E protein of JEV, were elicited by the DNA vaccine (Konishi *et al*., 1998).

In a subsequent study with pcDNA3JEME, the investigators measured anamnestic responses of vaccinated mice after challenge with JEV. They found that mice that developed high levels of neutralizing antibodies after challenge were more likely to survive than those that did not, suggesting that this anamnestic response is critical for protective immunity elicited by vaccination (Konishi *et al*., 1999).

The pcDNA3JEME construct was further evaluated in swine and was compared to another construct, pNJEME, which expressed the same JEV genes, but was developed using a plasmid vector containing an added intron A sequence downstream of the CMV promoter and upstream of the eukaryotic initiation site (Konishi *et al*., 2000b). Groups of five pigs were inoculated twice by i.m. or i.d. routes with the DNA vaccine candidates or by subcutaneous (s.c.) inoculation of a commercial, formalin-inactivated veterinary JE vaccine (JEVAX-A). Antibody responses were assessed with hemagglutination inhibition (HAI) and PRNT. Neutralizing and HAI antibody titers of $\leq$1:160 were detected in pigs given 450 μg of either construct i.m. Low or undetectable neutralizing antibody responses were measured in pigs given JEVAX-A according to the manufacturer's directions. To examine the anamnestic

response to DNA vaccination, three pigs previously vaccinated with 450 μg of pNJEME and three pigs previously vaccinated twice with JEVAX-A were boosted with JEVAX-A at day 116 of the study. The DNA-vaccinated pigs developed HAI titers as high as 1:2560 8 days after boosting, whereas the JEVAX-A pigs developed titers of 1:80. The authors conclude that the DNA vaccines elicit virus-specific memory B cells in swine and that the immune response is superior to that elicited by a commercial, veterinary vaccine (Konishi *et al.*, 2000b). Challenge studies are needed to determine if protection is also superior.

Another group prepared similar constructs by cloning the prM and E genes of JEV into pcDNA3 or plasmids derived from pcDNA3; however, the investigators optimized the nucleotide sequences surrounding the translation initiation site (Kozak, 1987) and modified and extended the signal peptide preceding prM sequences (Chang *et al.*, 2000). The constructs were used to vaccinate groups of five 3-week-old or 3-day-old ICR mice by i.m. injection. Some of the 3-week-old mice received a second vaccination 3 weeks later. All mice seroconverted by 3 weeks after vaccination and by 9 weeks had antibody levels $\geq$1600 with ELISA and from 1:40–1:160 with $PRNT_{90}$. Antibody remained detectable with ELISA throughout the 60-week observation period. The 3-day-old vaccinated mice also seroconverted, but had lower antibody titers than the older mice when measured by ELISA. The vaccinated neonatal mice were challenged at 7 weeks after vaccination by i.p. injection of JEV. All of the vaccinated mice survived, whereas 30–40% of control mice survived (Chang *et al.*, 2000). The investigators suggest that the improved protein synthesis and translocation elements introduced into their constructs led to enhanced secretion and formation of extracellular particles, which in turn elicited higher neutralizing antibody responses. That assumption is reasonable; however, no formal proof that extracellular particles were being formed in cells transfected with the DNA vaccines was given.

In studies by another group, two different expression plasmids were constructed to test the possibility that each might generate a different type of immune response in mice (Kaur *et al.*, 2002). Both constructs encoded prM. One plasmid encoded full-length JEV E protein, which the authors referred to as membrane-anchored E (prM-Ea) because it contained the C-terminal hydrophobic domain. The other plasmid encoded the N-terminal 80% of the E protein without the C-terminal membrane-anchoring domain, a form that the authors referred to as the secretory form of E protein (prM-Es). Both prM and E were detected in cells transfected with both constructs, but the proteins

were only detected in supernatants of cells transfected with prM-Es. These results contrast with those reported for DEN-1 (Raviprakash *et al.*, 2000a), where expression of the DEN-1 ME100 construct in 293 cells led to the production of extracellular VLPs and expression of the DEN-1 ME80 construct led to a degraded protein product. Perhaps these different results can be explained by differences between DENV and JEV or, more likely, subtle differences in the plasmid DNA constructs, for example in the leader sequences.

To test the immunogenicity of the constructs, the investigators inoculated mice with each plasmid delivered by i.m. injection or by gene gun. After primary inoculation, booster inoculations were administered on days 21 and 36. The vaccinated mice made antibodies that reacted against JEV and purified E protein as detected by ELISA on day 21. However, antibody titers against purified E protein were lower in mice inoculated by gene gun, but this may have been due to the dose of DNA administered, which was not clearly specified. As observed by others, antibody isotype analysis demonstrated that the ratio of IgG1 to IgG2a was higher when the gene gun was used for vaccination, whereas, this was reversed when i.m. injection was used, especially with the prM-Ea vaccine construct. Nevertheless, JEV-neutralizing antibody titers, thought to be a primary correlate of protection, were almost the same regardless of the plasmid vaccine used or the route of inoculation. Cell-mediated immune responses were also assessed in vaccinated mice by assaying for IL-4 and IFN-γ production, and by lymphocyte proliferation and CTL assays. Spleen cells from animals inoculated by gene gun made higher levels of IL-4 than cells from animals inoculated i.m. Conversely, cells from animals inoculated i.m. produced higher levels of IFN-γ, particularly when the prM-Ea construct was used for the inoculations. Lymphocytes from all vaccinated mice proliferated in vitro to varying degrees when exposed to JEV E antigen. However, there was so much animal-to-animal variation that no one vaccine or delivery route was clearly superior. CTL activity measured by lysis of target cells in vitro was more uniform, but only at the highest E:T ratio (i.e., 200) was there a difference to suggest a marginal superiority of i.m. inoculation over gene gun. Finally, in protection experiments, 50–60% survival rates (versus 10% in controls) were seen in mice vaccinated with prM-Ea or prM-Es administered by gene gun or i.m. inoculation, with no significant difference in protection among the vaccine groups. In contrast, over 90% of mice vaccinated with inactivated JEV, used as a positive control in these experiments, survived challenge. Although the mice vaccinated with inactivated virus had lower CMI responses, they had higher neutralizing antibody

titers than the animals that received DNA vaccines, perhaps reaffirming the importance of neutralizing antibody in protection against JEV. Adoptive transfer of E-specific antibodies, but not crude or T-cell–enriched splenocytes to sublethally irradiated mice conferred protection, consistent with the importance of the humoral response to a DNA vaccine for immunity to JEV challenge (Pan *et al.*, 2001).

Although neutralizing antibodies to E are likely the main mechanism of protective immunity for JEV, protection can be achieved without good neutralizing, or even without good antibody responses. An example of this was observed by investigators who constructed a plasmid expressing an intracellular form of E (Ashok and Rangarajan, 1999). Outbred Swiss mice were injected i.m. with 50 μg of the construct at days 0 and 15 or were inoculated intranasally (i.n.) with 20 μg of the DNA 5 times at weekly intervals. The mice were challenged by i.c. injection of JEV 2 weeks after vaccination. About half of the mice in each group survived challenge, whereas none survived when given a control plasmid. The investigators could detect no JEV-specific antibodies by ELISA before or after challenge. In contrast, they measured low levels of lymphocyte proliferative activity after vaccination by both routes, and large amounts of activity after challenge (Ashok and Rangarajan, 1999). The absence of mRNA for IL-4 and the increase in mRNA of (IFN-γ), as measured by quantitative PCR, suggest that the DNA vaccine elicited a Th-1 type response. The authors conclude that the construct they made elicits a poor humoral immune response, but a good cell-mediated response, which was apparently sufficient to partially protect mice from i.c. challenge with JEV.

In a subsequent study, these authors generated secretory forms of complete E or truncated E by introducing a tissue plasminogen activator (TPA) signal sequence preceding the JEV E coding regions (Ashok and Rangarajan, 2002). Comparing the nonsecretory and secretory constructs in mice revealed that none of them elicited good neutralizing antibody responses either before or after i.c. challenge. Of the DNAs examined, the truncated E construct with the TPA signal sequence was found to confer the best protective immunity (12 of 17 mice were protected). Although results from other studies, described above (Konishi *et al.*, 1999, 2000a, 2000b), indicated that an anamnestic antibody response was important for protection from peripheral challenge with JEV or DENV, the authors of this study suggest that protection from i.c. challenge is not dependent on such a response, but rather that cell-mediated immune responses are more important than humoral responses (Ashok and Rangarajan, 2002). Because direct i.c. challenge is not a natural route of JEV infection, it is not clear

how relevant these findings are for developing a human vaccine for JEV.

IV. DNA Vaccines for Tick-Borne Encephalitis

Candidate DNA vaccines for tick-borne encephalitis have been assessed in mouse challenge models and in monkeys. One group prepared DNA vaccines expressing the prM and E genes of Russian spring-summer encephalitis virus (RSSEV), or Central European encephalitis virus (CEEV) and evaluated their ability to provide homologous and cross-protective immunity in BALB/c mice (Schmaljohn *et al.*, 1997). Adult mice given two vaccinations, 4 weeks apart by gene-gun inoculation of approximately 1 μg of either DNA vaccine or a combination of both vaccines, developed neutralizing antibodies and were protected from challenge with either virus. Challenged mice showed little or no rise in antibody titer, suggesting sterile immunity. The duration of protective immunity of the CEEV DNA vaccine was also measured in mice. Adult mice were vaccinated by gene gun one, two, or three times at various intervals and challenged from 1 to 52 weeks later. A single dose of DNA protected all mice from challenge at 8 weeks after vaccination and two or three doses given at 4-week intervals protected all mice from challenge 1 year after the initial vaccination (Schmaljohn *et al.*, 1997).

The same RSSEV and CEEV DNA vaccine constructs were further evaluated in monkeys (Schmaljohn *et al.*, 1999). Groups of four rhesus macaques were vaccinated by gene gun with approximately 10 μg of the RSSEV DNA, the CEEV DNA, both DNAs or by i.m. injection of a formalin-inactivated CEEV human vaccine. Each vaccine was delivered on days 0, 30, and 70 and blood samples were collected and analyzed for antibody responses with ELISA and PRNT. All monkeys except for one receiving the RSSEV DNA vaccine developed $PRNT_{80}$ titers ranging from 1:1280 to greater than 1:20480 when assayed 2 weeks after the third vaccination. Fifteen weeks after vaccination, neutralizing antibodies had dropped but were still $>$1:160 for all monkeys except the one that initially had a low titer. Boosting the monkeys with 2.5 μg of DNA or 10 μg of DNA resulted in increases in the neutralizing antibody response to levels equivalent to those observed after the initial series. Because monkeys, like humans, do not always become ill when infected with RSSEV or CEEV, instead of challenging the monkeys, sera from the monkeys were passively transferred to mice and mice were challenged with RSSEV or CEEV. Transfer

of 50 μL of monkey sera completely protected all mice, and transfer of 10 μL resulted in protection that correlated with the initial neutralizing titer of the transferred monkey serum. That is, sera from monkeys with the highest neutralizing antibody titers offered complete protection from challenge with either RSSEV or CEEV whereas lower-titer sera protected fewer mice. These studies demonstrate that a vaccine based on the prM and E genes of either RSSEV or CEEV might be useful as a human vaccine for TBE.

In another study, investigators constructed candidate DNA vaccines expressing CEEV full-length E, or truncated E with or without prM (Aberle *et al.*, 1999). Groups of 16 BALB/c mice were vaccinated twice at 4-week intervals by i.m. injection of 100 μg of DNA or by gene-gun inoculation of 2 μg of DNA. All mice vaccinated by either method with the complete prM and E DNA developed neutralizing antibodies and were protected from challenge, whereas no neutralizing antibodies and low or no protection was observed in mice given the other candidate DNA vaccines (Aberle *et al.*, 1999). The types of immune responses that these DNA vaccines elicited were further examined by measuring the antibody isotype profiles of the vaccinated mice. Although the gene-gun vaccinated mice displayed a predominantly Th-2 type response, as indicated by a higher ratio of IgG1:IgG2a, and the i.m. vaccinated mice had a predominantly Th-1 response, mice given the complete prM and E construct had more balanced Th-1/Th-2 responses. The investigators concluded that not only the method of delivery, but also the antigen being expressed influences the type of response elicited (Aberle *et al.*, 1999).

In addition to studies evaluating mouse protection with prM and E gene constructs, studies to assess protection by an E-NS1 construct or an NS3 construct of RSSEV were reported (Mitrofanova *et al.*, 1997; Morozova *et al.*, 1999, 2000). The NS3 construct was prepared using a pcDNAI vector, whereas the E-NS1 construct was generated using a plasmid with an SV40 promoter (psVK3), rather than a CMV promoter. BALB/c mice, vaccinated by i.m. injection of 80–100 μg of the E-NS1 DNA, five times over 2 months, were completely protected from disease and death, whereas all control mice became sick and about half died after challenge with homologous virus. No immune response data were reported (Mitrofanova *et al.*, 1997). The NS3 construct was assessed for antibody response and protection by giving BALB/c mice four i.m. injections of 100 μg of the construct at weekly intervals. Antibody responses of 1:50 by Western blot and 1:10–1:100 by ELISA were observed and none of the mice was protected from challenge with RSSEV (Mitrofanova *et al.*, 1997).

V. DNA Vaccines for West Nile Encephalitis

With the recent spread of West Nile virus (WNV) throughout the United States, vaccine development for this disease has gained momentum. Two DNA vaccine studies have been reported, although other efforts are likely under way. A candidate DNA vaccine expressing the prM and E genes of WNV controlled by a CMV promoter (pCBWN) was evaluated for protection of mice and horses (Davis *et al.*, 2001). Groups of 10 3-week-old ICR mice were vaccinated once by i.m. injection of 100 μg of pCBWN. All mice developed IgG ELISA antibody titers of 1:640–1:1280 3 weeks after vaccination. Pooled sera from mice revealed neutralizing antibody titers of 1:80 when assayed 3 and 6 weeks after vaccination and 1:320 or 1:640 at 9 weeks after vaccination. All of the vaccinated mice survived challenge by i.p. injection of WNV or by exposure to the bite of virus-infected mosquitoes, whereas none of the control mice that received a similar plasmid expressing green fluorescent protein survived challenge. A second experiment was performed comparing vaccination by i.m. injection of 100 μg of the construct and injection of 10 μg, 1 μg, or 0.1 μg of the pCBWN DNA vaccine followed immediately by the use of an electroporation device to increase DNA transfer into the host cells (Mir *et al.*, 1999). Pooled sera analyzed at 6 weeks after vaccination showed neutralizing antibody titers ($PRNT_{90}$) of 1:160 in the 100-μg and 10-μg dose groups and 1:80 or 1:40 in the 1-μg or 0.1-μg dose groups. All mice survived i.p. challenge with WNV, whereas none of the control mice survived (Davis *et al.*, 2001).

The pcBWN DNA vaccine was further evaluated in four horses that were vaccinated i.m. with a single dose of 1 mg of DNA and were challenged by exposure to WNV-infected mosquitoes on day 39. Two days before challenge, $PRNT_{90}$ titers of the horses were 1:5, 1:20, 1:20, and 1:40. After challenge, none of the vaccinated horses displayed viremia or fever, while seven of eight unvaccinated control horses became viremic. The vaccinated horses had gradual rises in neutralizing antibodies over the 14-day postchallenge observation period. Similar postchallenge antibody levels were measured in the control horses, thus the vaccinated horses appear to have been protected from a rapid replication of challenge virus that would have triggered an anamnestic antibody response. These data provide encouragement for the use of DNA vaccines to protect large animals with a single vaccine dose.

A different approach was tested for WNV DNA vaccination by a group who expressed the viral capsid gene using pcDNA3 (Yang *et al.*, 2001). BALB/c mice vaccinated by i.m. injection of the construct

developed T cell responses as indicated by expression of the Th1-type cytokines (IFN-γ) and IL-2, and a CTL response. Because the mice were not challenged with WNV, it is not known whether a vaccine such as this, which elicits predominantly cellular immune responses, would offer protective immunity.

VI. DNA Vaccines for Other Flavivirus Diseases

Studies on DNA vaccines for three additional flaviviruses, St. Louis encephalitis (SLE), Murray Valley encephalitis (MVE), and Louping Ill (LI) were reported. The candidate DNA vaccine for SLE virus was constructed by inserting the preM and E genes into the plasmid vector pMV100 under control of the CMV immediate early promoter (Phillpotts *et al.*, 1996). *In vitro* transfection of Vero cells followed by immunofluorescence staining confirmed expression of the SLE-specific proteins but no data were presented as to whether or not the protein was secreted. Porton TO strain mice were inoculated i.m. either once with 50 μg or twice with the second dose consisting of 100 μg given on day 21. The animals were then challenged with 30 LD_{50} of SLE at day 49. Seventy-five percent of the animals that received a single injection survived the challenge (9/12) compared to 57% of those that received two injections (4/7). The authors reported no statistically significant difference in survival between the two groups. Animals that received phosphate-buffered saline and the vector without insert had survival rates of 9% and 25%, respectively. Serum samples obtained 7 days before challenge failed to show the presence of neutralizing antibody but did demonstrate immunoreactivity in ELISA with SLE antigen derived from Vero cell culture fluid. The authors attribute the inability to detect neutralizing antibody activity to the use of relatively high dilutions of sera.

The MVE candidate vaccine was constructed by inserting the viral prM and E genes into pCDNA1 or pCDNA3 (Colombage *et al.*, 1998). In one experiment, BALB/c, CBA/H and C57Bl/6 mice were vaccinated by two gene-gun inoculations 0.5–2 μg of DNA 4 weeks apart. Neutralizing antibody titers ($PRNT_{50}$) ranging from 1:80 to 1:320 were measured after vaccination and before challenge. Mice were challenged at various times (from 7 to 26 weeks) after vaccination by i.p. injection of 10^8 plaque-forming units (pfu) of MVEV. All vaccinated mice survived challenge, whereas 40% of the BALB/c mice, 40–50% of the C57Bl/6 mice, or 33–80% of the CBA/H mice that were naïve or were injected with a pDCNA control plasmid survived the challenge.

In a second experiment, BALB/c mice were vaccinated by i.m. injection of 100–125 μg of the DNA twice at a 6-week interval or four times at 3- to 4-week intervals. Only at weeks 14 to 15 after vaccination, and after four vaccinations did nine of 10 mice in the two groups show MVE-specific antibodies by ELISA, and none of the mice had detectable neutralizing antibody responses. Nevertheless, upon i.p. challenge, eight of 10 mice vaccinated four times with the prM and E construct survived challenge (Colombage *et al.*, 1998).

As reported by others, the type of antibody isotype elicited by the DNA vaccines in BALB/c mice was related to the method of delivery with i.m. inoculation inducing IgG2a responses (Th-1 type) and gene-gun–inducing IgG1 (Th-2 type) responses. In the other mouse strains, the same trend was seen, although the predominance of IgG1 in gene-gun vaccinated mice was less pronounced. In general, these studies support the contention that gene-gun vaccination is superior to i.m. injection for eliciting neutralizing antibody responses.

Candidate DNA vaccines for LI were constructed by inserting the viral prM and E, or NS1 genes into 2 different types of plasmids (Fleeton *et al.*, 2000). One plasmid had the genes under control of the CMV promoter, while in the other, the CMV promoter drives expression of an alphavirus replicon containing the LI genes. Translation of the alphavirus replicon RNA in cells produces the alphavirus replicase complex, which results in self-amplification of the recombinant RNA encoding the LI proteins. BALB/c mice were vaccinated two times, 3 weeks apart by i.m. injection of 50 μg, 5 μg, or 1 μg of each plasmid. All mice developed antibodies detected with ELISA except for three mice in the groups that received 5 μg or 1 μg of the replicon/DNA construct expressing NS1. Comparing 50-μg doses of the DNA vaccines in C57Bl/6 mice and BALB/c mice showed that the C57Bl/6 mice developed slightly higher ELISA antibody responses. A fluorescent focus-reduction assay was used to demonstrate that both DNA vaccines elicited low levels of neutralizing antibodies. To evaluate protection, BALB/c or C57Bl/6 mice were vaccinated once or twice with the DNA vaccines then challenged with three different strains of LIV. Only partial protection was elicited by the various DNA vaccines. The NS1 vaccines elicited little or no protection. The prM and E constructs or combinations of the prM and E and the NS1 constructs elicited partial protection from challenge with two of the LIV strains. No protection was provided to challenge by the third strain of LIV. Neither the standard DNA vaccine nor the replicon-based DNA vaccine elicited protective immunity equivalent to that obtained using a packaged alphavirus replicon expressing the LIV genes. Although unexplained in the

report, the poor performance of the replicon/DNA vaccine contrasts with results reported using a similar vector to provide improved immune responses to *Herpes simplex* virus as compared to that elicited by a standard DNA vaccine (Hariharan *et al.*, 1998).

VII. Conclusion

Results of studies on DNA vaccines for flaviviruses reveal few surprises with respect to their immunogenicity. As with other vaccine methods, the E protein is the most important immunogen, and the presence of prM, where studied, improves vaccine efficacy. Neutralizing antibodies are generally the desired vaccine response and their presence correlates with protective immunity. Although direct comparison of the various DNA vaccine delivery methods has not been reported, gene-gun vaccination appears to be the most effective means of eliciting high levels of neutralizing antibodies with flavivirus DNA vaccines. Presumably, these results related to the ability of gene-gun delivery to efficiently stimulate antigen-presenting cells that are present in the skin. Drawbacks to gene-gun delivery include technically difficult manufacturing methods and the need to give several doses of vaccine. It remains to be determined if these obstacles can be overcome by alternative transcutaneous delivery methods such as microneedle injection, abrasion or chemical transport methods. In addition, methods involving incorporation of the DNA in liposomes or other particulate media are all being developed and should be compared to skin delivery and injection in aqueous solutions. Most importantly, a clinical proof of concept is needed to further the use of DNA vaccines for flaviviruses.

A major advantage of DNA vaccines is their potential to be used in combination for a number of diseases. This promise has not yet been tested clinically. An interesting offshoot of the combination DNA vaccine concept involves "gene-shuffling" method (Whalen *et al.*, 2001). With this method, it may be possible to blend the DNAs of genetically similar flaviviruses to yield a single chimeric gene that would elicit protective immune responses to all of the viruses. As yet, this approach has not been validated for flaviviruses.

In summary, although DNA vaccination is a relatively new approach for preventing flaviviral diseases, numerous DNA vaccines and delivery approaches have achieved reasonable success in the laboratory and will likely be put to the clinical test very soon.

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CHIMERIC FLAVIVIRUSES: NOVEL VACCINES AGAINST DENGUE FEVER, TICK-BORNE ENCEPHALITIS, AND JAPANESE ENCEPHALITIS

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I. Introduction

The flavivirus genus of the Flaviviridae family contains approximately 70 viruses, most of which are arthropod-borne and have been divided into antigenic complexes and sub-complexes on the basis of serological data, vector transmissibility and phylogenic analysis (Calisher *et al.*, 1989; Kuno *et al.*, 1998; Monath and Heinz, 1996). Many of the arthropod-borne flaviviruses are important human pathogens responsible for diverse illnesses, including yellow fever (YF), Japanese encephalitis (JE), tick-borne encephalitis (TBE), and dengue fever. A live-attenuated YF virus vaccine, strain 17D, has been developed by serial passage of the wild type virus strain Asibi in animals and cultured cells. The live YF virus vaccine is used extensively throughout the world and has an impressive safety and efficacy record (reviewed by Monath, 1999). The YF 17D vaccine is the paradigm for other live attenuated flavivirus vaccines. Live-attenuated vaccines offer the potential advantages of a single dose, delivering the complete repertoire of antigens that closely resemble the pathogen against which they are meant to protect. The live vaccine may induce a cytokine milieu similar to that following natural infection, stimulating innate immunity and induction of strong immunologic memory and a complete and long-lasting humoral and cell-mediated response. Attempts to develop safe and effective live-attenuated vaccines against dengue and tick-borne encephalitis have not yielded licensed products and a live-attenuated vaccine against Japanese encephalitis is approved for use only in China. Advances in the development of new vaccines against dengue and Japanese encephalitis have been reviewed recently (Barrett, 1997; Kinney and Huang, 2001).

This chapter describes the rational design of recombinant flavivirus vaccines, based on modification of their molecular structure by creating chimeric viruses. The molecular reorganization of flaviviruses to achieve this purpose depends on a basic understanding of the flavivirus genome organization and the role of viral proteins in immunity. These concepts are dealt with in detail in other chapters of this book, and are reviewed only briefly here. The positive-strand RNA genome of the arthropod-borne flaviviruses contains a 5′ noncoding region (NCR) followed by the genes for three structural proteins, i.e., capsid (C), pre-membrane (prM), and envelope (E) and seven nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 and a 3′ NCR (reviewed by Chambers *et al.*, 1990). The 5′ and 3′ NCRs include features of conserved primary sequences and secondary stem-and-loop structures (Brinton and Dispoto, 1988; Hahn *et al.*, 1987). The

structural and nonstructural proteins are cleaved from a long polyprotein by a combination of cellular and viral proteases. The flaviviruses contain an internal nucleocapsid that is surrounded by the M and E proteins embedded in a cell-derived lipid envelope. The E protein interacts with the prM, or its cleaved product, M, during virus maturation and entry into host cells (Heinz *et al.*, 1994; Stadler *et al.*, 1997). The three-dimensional structure of the TBEV envelope (E) glycoprotein has been determined at 2Å resolution (Rey *et al.*, 1995). Evidence indicates that the nonstructural proteins NS1, NS2A, NS2B and NS3 play a role in polyprotein cleavage (Bazan and Fletterick, 1989; Falgout *et al.*, 1989, 1991; Gorbalenya *et al.*, 1989; Hori and Lai, 1990; Lain *et al.*, 1989). Nonstructural proteins NS1, NS3, NS4A, and NS5 are involved in various steps of RNA replication (Lindenbach *et al.*, 1997; Muylaert *et al.*, 1997; Steffens *et al.*, 1999; Tan *et al.*, 1996; Wengler and Wengler, 1993). The capacity of viral replication in the infected host is likely determined by the functional activity of each of the structural and nonstructural proteins and their intricate interactions. Neutralizing antibodies specific for the prM/M and E structural antigens provide the first line of host defense against these viruses. Immunization with the NS1 protein and passive transfer of NS1-specific antibodies also protect animals against flavivirus infections, presumably by a mechanism of complement-dependent cytolysis (Falgout *et al.*, 1990; Schlesinger *et al.*, 1986, 1988). Flavivirus infections also induce the response of cytotoxic T lymphocyte cells that recognize the structural proteins as well as several nonstructural proteins in a flavivirus type-specific or cross-reactive manner (Bukowski *et al.*, 1989; Kurane and Ennis, 1992; Livingston *et al.*, 1994; Rothman *et al.*, 1996).

A. Dengue

Currently, the four dengue virus serotypes (DEN1-4) are the most important flaviviruses in terms of human morbidity, which is estimated to involve up to 100 million people every year (Gubler, 1998; Monath, 1994). Dengue epidemics pose a major public health problem in tropical and subtropical regions where *Aedes aegypti* and (to a lesser extent) *Aedes albopictus* mosquito vectors are abundant. Dengue illness ranges from mild dengue fever (DF) to severe dengue, characterized by hemorrhage and shock (dengue hemorrhagic fever/dengue shock syndrome, or DHF/DSS), which, if untreated, has a high case-fatality rate, especially in children younger than 15 years of age (Halstead, 1988). Most DHF/DSS cases are in individuals who have

been previously infected with a different dengue virus serotype. It is believed that immunopathological mechanisms involving antibody enhancement of viral replication as well as cytotoxic T-lymphocyte–mediated cytokines may contribute to severe dengue disease (Halstead, 1970; Rothman and Ennis, 1999). Highly virulent dengue virus strains could also cause severe illness during primary infection (Leitmeyer *et al.*, 1999; Mangada and Igarashi, 1998; Rosen, 1977).

The mechanism of DHF/DSS remains controversial. Immunity induced following dengue virus infection is thought to be lifelong against the same dengue virus serotype. Cross-protective immunity against other dengue virus serotypes is believed to be brief, lasting up to 6 months (Sabin, 1952), although more recent evidence suggests that heterologous cross-protection may be more important than previously thought and could serve as a filter for emergence of more virulent strains. Because of the immunopathologic role of heterologous antibody and cross-reactive T killer cells, a tetravalent vaccine is required to induce protective immunity against all four dengue virus serotypes and to minimize the risk of sensitizing vaccinees for severe dengue. The goal is simultaneous immunization against all four serotypes, thus leaving the immunized host nonsusceptible to wild-type dengue infections. If two or more doses are required to achieve complete solid immunity against the full repertoire of dengue serotypes, as may be the case for empirically derived live attenuated vaccines (Saluzzo, 2003) it will be essential to show, in appropriately powered clinical trials, that incompletely immunized subjects are not at risk of DHF/DSS.

Early attempts to develop live, attenuated dengue vaccines used empirical methods. Wild type dengue viruses were serially passaged in suckling mouse brains to adapt the virus to grow efficiently (Hotta, 1952; Sabin and Schlesinger, 1945). This adaptive process selected for mouse neurovirulent mutants that also exhibited attenuation for humans (Hotta, 1952; Sabin and Schlesinger, 1945; Wisseman *et al.*, 1963). These dengue virus mutants were not developed further as potential vaccines because extensive virus purification would be required to remove contaminating mouse brain antigens.

Subsequently, serial passage in cultured cells was employed to attenuate the wild type dengue viruses (Saluzzo, 2003). Candidate vaccines have been identified for each of the four dengue serotypes and tested in clinical trials involving a limited number of volunteers (reviewed by Kanesa-thasan *et al.*, 1997; and by Bhamarapravati and Yoksan, 1997). However, reactogenicity and immunogenicity of these candidate vaccines, especially as multivalent combinations, were not

uniformly satisfactory (Bhamarapravati and Sutee, 2000; Kanesathasan *et al.*, 2001). Development of a live dengue virus vaccine against all four dengue virus serotypes remains an elusive goal.

B. Japanese Encephalitis

Japanese encephalitis virus (JEV) is the leading cause of epidemic viral encephalitis throughout Asia and in parts of Australia, with approximately 16,000 cases reported every year. JE is a severe disease with a fatality rate as high as 20% and a large proportion of the survivors exhibits neurological sequelae (reviewed by Tsai, 2000). JEV is transmitted principally between Culex mosquitoes, pigs, and birds. Humans and horses are incidental hosts, but are susceptible to illness. A formalin-inactivated vaccine (manufactured by BIKEN, Osaka, Japan) prepared from JEV grown in mouse brains is licensed internationally (Takaku *et al.*, 1968). The vaccine is moderately effective and requires repeated immunizations to achieve a satisfactory level of immunity. Adverse effects of hypersensitivity and neurological reactions have been associated with the inactivated JE vaccine (Ohtaki *et al.*, 1995; Plesner *et al.*, 2000). A live JEV vaccine, strain SA14-14-2, attenuated by serial passage in mice, hamsters, and primary hamster kidney cell culture, has been used for immunization in China (Liu *et al.*, 1997). The live JEV vaccine is safe and effective, but requires multiple doses for full protection (Hennessy *et al.*, 1996). There is also a concern about the vaccine manufacturing practices, including the use of the cell substrate, which has not been certified. An improved, single-dose live JE vaccine that is produced in an acceptable cell substrate and capable of inducing long-lasting immunity without adverse reactions would be highly desirable to replace the current products.

The mosquito-borne JE antigenic complex also includes JE, St. Louis encephalitis, Murray Valley encephalitis (MVE), Kunjin, West Nile, Usutu, Kokobera, Stratford, Alfuy, and Koutango viruses. West Nile virus was first isolated in Africa, and was subsequently found in the Middle East, Europe, Asia, and Australia, and most recently in North America (Hall, 2000; Hubalek, 2000). West Nile virus is now regarded as an emerging virus of public health concern in many countries.

C. Tick-Borne Encephalitis

The tick-borne encephalitis (TBE) antigenic complex includes Russian spring-summer encephalitis virus (RSSV) or the Far Eastern subtype, and Central European encephalitis (CEE) virus, Langat, louping

ill (LI), Kyasanur Forest disease (KFD), Negishi, Omsk hemorrhagic fever (OHF), Powassan (POW), Karshi, and Royal Farm viruses (Calisher *et al.*, 1989; Clarke, 1964). The most important pathogens (RSSE, CEE, POW, LI, and OHF) are endemic throughout most of the Northern Hemisphere. In tropical regions, Langat virus was isolated from ticks in Malaysia and did not appear to be associated with naturally acquired human disease (Smith, 1956), while KHD is a human pathogen from India. Case fatality rates from RSSV infections are reported to be as high as 30%, compared to the rates of 1–5% in the case of CEE (Calisher, 1988), but this difference in severity may in fact be due to reporting artifacts.

TBE complex viruses share E glycoprotein cross-reactive epitopes and can induce cross-resistance to each other (Chiba *et al.*, 1999; Holzmann *et al.*, 1992). A highly purified, formalin-inactivated vaccine prepared from the CEE virus grown in chick embryo cells (known as FSME-Immun vaccine) is licensed for use in Europe (Heinz *et al.*, 1980). This inactivated vaccine requires multiple immunizations to achieve a satisfactory level of protective efficacy. The vaccine is presumed to cross-protect against the closely related RSSE subtype, but human data are lacking on this point. A single-dose, live TBE vaccine that is safe and capable of inducing a long-lasting immunity would represent an improvement over the existing vaccine.

II. Flavivirus Full-Length cDNA Clones and Attenuating Mutations

A. Molecular Basis of Attenuation of Empirically Derived Vaccines

Genome analyses of flavivirus vaccine strains have partially revealed the molecular basis of attenuation, leading to a better understanding of pathogenesis. These analyses have revealed multiple mutations generally distributed throughout the structural and nonstructural regions of the genome of YF 17D (Hahn *et al.*, 1987), JEV SA14-14-2 (Nitayaphan *et al.*, 1990), DEN1 45AZ5 PDK-27 (Puri *et al.*, 1997), DEN2 PDK-53 (Kinney *et al.*, 1997), and DEN1 PDK-13 (Huang *et al.*, 2000). Many viral functions could contribute to the attenuation phenotype. These vaccine viruses do not appear to share a common attenuating mutation. Exploration of the role of putative attenuation determinants has been made possible by site directed mutagenesis of infectious clones.

B. Flavivirus Infectious Clones

Infectious RNA transcripts derived from cDNA of the attenuated YF 17D vaccine were first prepared using a two-plasmid system by cloning the 5′ and 3′ halves of the genome separately and then ligating them in vitro prior to transcription (Rice *et al.*, 1989). Instability of full-length cDNA in *Escherichia coli* proved to be an impediment for several other flaviviruses. The two-plasmid system was also used to obtain the infectious RNA transcripts of Japanese encephalitis virus strain JaOArS982 (Sumiyoshi *et al.*, 1992). To accelerate molecular studies and development of vaccines against dengue, a stable full-length cDNA copy of wild type DEN4 strain 814669 was first cloned in *E. coli* strain BD1528 for transcription of infectious RNA (Lai *et al.*, 1991). Full-length cDNA clones of the DEN2 New Guinea C (NGC) strain and DEN1 Western Pacific (WP) 74 strain were assembled in yeast and then propagated in *E. coli* using a shuttle vector (Polo *et al.*, 1997; Puri *et al.*, 2000). The full-length cDNA clones of several other flaviviruses have been constructed, including DEN2 NGC (Gualano *et al.*, 1998; Kapoor *et al.*, 1995), candidate vaccine DEN2 PDK-53 and its DEN2 16681 parent (Kinney *et al.*, 1997), Kunjin strain MRM 61C (Khromykh and Westaway, 1994), MVEV prototype strain 1-51 (Hurrelbrink *et al.*, 1999), TBEV Neudoerfl and Hypr strains (Mandl *et al.*, 1997), Langat virus prototype TP21 and attenuated E5 strains (Campbell and Pletnev, 2000; Pletnev, 2001), and West Nile virus strain WN956 (Yamshchikov *et al.*, 2001).

The availability of flavivirus cDNA clones has made it possible to introduce mutations into the viral genomes by site-directed mutagenesis and directly analyze the effects of the mutations on the phenotype of the recovered viruses (reviewed by Ruggli and Rice, 1999). The menu of flavivirus mutations that decrease the efficiency of viral replication in cultured cells or in animals and could be evaluated as candidate attenuating mutations is extensive. As an example, genetically defined mutants have been constructed for YF (Muylaert *et al.*, 1997), DEN4 (Lai *et al.*, 1992), DEN2 (Matusan *et al.*, 2001), JEV (Arroyo *et al.*, 2001; Sumiyoshi *et al.*, 1995), and TBEV (Mandl *et al.*, 2000). Flavivirus mutants containing deletions engineered into the 3′ and 5′ NCRs exhibited attenuation characteristics both in cell culture and in primates (Cahour *et al.*, 1995; Mandl *et al.*, 1998; Men *et al.*, 1996; Zeng *et al.*, 1998). Mutations in the hinge region spanning domains I and II of the E glycoprotein (Arroyo *et al.*, 2001a; Hurrelbrink and McMinn, 2001; Sumiyoshi *et al.*, 1995) and in the upper-lateral surface of domain III (Mandl *et al.*, 2000) have been shown to reduce flavivirus virulence.

Recombinant DNA technology has made it possible to pursue the novel strategy of creating chimeric viruses, in which the structural protein genes of one flavivirus are replaced by the corresponding genes of another flavivirus. The sequences of the wild type DEN4, DEN2 PDK-53 candidate vaccine, and YF 17D vaccine strains have been explored as vectors. Chimeric flaviviruses are in development as candidate live-attenuated vaccines against dengue, Japanese encephalitis, West Nile, and tick-borne encephalitis viruses (Table I).

Infectious clone technology has many advantages for manufacture of live-attenuated vaccines in mammalian cell culture. The manufacturing method begins with bacterial plasmids from which cDNA is recovered, transcribed to RNA, and electroporated into an acceptable cell line for manufacture. This reduces the likelihood of contamination with adventitious viruses. Moreover, the recombinant vaccine virus is clonal and can be precisely defined by nucleotide sequencing for quality control and assessment of genomic stability.

III. Chimeric Flaviviruses against Dengue Using DEN4 as the Backbone

A. *Intertypic Chimeric Dengue Viruses*

The full-length cDNA clone of wild type DEN4 strain 814669 was first used to construct viable chimeric dengue viruses in which the C-prM-E genes of DEN4 are replaced by the corresponding genes of another dengue serotype virus (Bray and Lai, 1991; Lai *et al.*, 2001). Replacement of genes is facilitated by the fact that there is significant sequence conservation among the four dengue serotype viruses. Initially, the dengue intertypic full-length cDNA clones were constructed between the C-prM-E genes of the prototype DEN1, Western Pacific strain (WP), or the mouse-adapted DEN2, New Guinea C strain (NGC) and the remaining sequence from the DEN4 virus (Fig. 1). The chimeric genome construction used a dengue virus–conserved Bgl II site (agatct) in the 5′UTR and an Xho I site introduced near the end of the DEN4 E gene without altering the amino acid sequence N-S-R. The RNA transcripts of the chimeric DEN4/DEN1 or DEN4/DEN2 DNA construct yielded viable viruses in transfected simian LLC-MK_2 cells, a permissive cell line for dengue virus replication. These chimeric viruses exhibited DEN1 or DEN2 serotype antigenicity. A chimeric DEN4/DEN3 virus expressing the C-prM-E genes of the wild type DEN3 strain H53489 was similarly constructed (Chen *et al.*, 1995).

The growth rate of the chimeric DEN4/DEN2 virus in LLC-MK_2 cells was reduced relative to that of the parental DEN4 or DEN2. The reduced growth phenotype of the DEN4/DEN2 chimera was interpreted as the result of chimerization due to the genetic incompatibility between the heterologous DEN2 C and the DEN4 backbone sequences (Bray and Lai, 1991). Construction of a chimeric DEN4/DEN2 virus that contains only the DEN2 prM-E genes was explored as an alternative strategy (Fig. 1). The progeny of DEN4/DEN2(prM-E) were readily recovered and grew to 10^6 pfu/mL in LLC-MK_2 cells or 10^7 pfu/mL in C6/36 cells (Bray *et al.*, 1996; Hiramatsu *et al.*, 1996).

B. Monkeys Immunized with Intertypic Dengue Chimeras Are Protected against Homotypic Dengue Virus Challenge

Research on dengue vaccines has been hampered by the lack of an appropriate animal model. Primates are the closest human surrogates for dengue virus infection (Rosen, 1958). Dengue virus–infected monkeys develop neutralizing antibodies, which play a major role in protective immunity. Monkeys infected with dengue virus also develop viremia similar to that observed in humans, but do not exhibit disease symptoms.

Rhesus monkeys in groups of four were inoculated by the subcutaneous (SC) route with 3×10^5 plaque-forming units (pfu) of DEN4/DEN1 (C-prM-E, WP), DEN4/DEN2 (prM-E, NGC), parental DEN4 814669, DEN1 WP, or DEN2 NGC, from which the chimeric viruses were derived (Bray *et al.*, 1996) (Table II). Viremia was detected in at least two of four monkeys immunized in each group. Monkeys immunized with the DEN4/DEN1 or DEN4/DEN2 chimera developed a homologous mean 50% plaque reduction neutralization ($PRNT_{50}$) titer of 320 or 640, respectively. Three of four monkeys infected with the DEN4/DEN1 chimera, and all four monkeys infected with the DEN4/DEN2 chimera were protected against homologous DEN1 or DEN2 challenge as shown by the complete absence of viremia. Nearly all monkeys previously immunized with DEN4 became viremic following cross-challenge with DEN1 or DEN2. Co-immunization of monkeys with a mixture of the DEN4/DEN1 and DEN4/DEN2 chimeras, each at 1.5×10^5 pfu/dose, elicited mean $PRNT_{50}$ titers of 293 against the DEN1 and 452 against DEN2 virus. These animals were completely protected against DEN1 or DEN2 challenge (Table II). Similar intertypic dengue chimeras could be used to express serotype-specific antigens in a live-attenuated tetravalent vaccine for humans.

TABLE I

Chimeric Flavivirus Vaccines

Target vaccine	Donor virus (strain), structural genes donated	Backbone	Laboratory/ company	Trade name	Status	Key references
Dengue	Dengue 1 (WP, wild-type) C-prM-E	Dengue 4 (814669, attenuated[a])	NIAID, NIH		Preclinical (monkeys); Phase 1 (DEN4 vector only)	Men *et al.*, 1996; Lai *et al.*, 2001
	Dengue 2 (NGC, wild-type) C-prM-E				Preclinical (monkeys)	
	Dengue 3 (H53489, wild-type) C-prM-E				Preclinical (monkeys)	
	Dengue 1 (16007 wild-type and PDK13, attenuated) C-prM-E	Dengue 2 (PDK53, attenuated)	CDC		Preclinical (mice)	Huang *et al.*, 2000
	Dengue 2 (PR159, wild-type) prM-E	YF 17D	Cal Tech		Preclinical (mice)	van der Most *et al.*, 2000
	Dengue 2 (NGC, wild-type)m prM-E	YF 17D	Oswaldo Cruz		Preclinical (mice)	Caufour *et al.*, 2001
	Dengue 1 (PUO359, wild-type) prM-E	YF 17D	Acambis Inc. (Aventis Pasteur)	ChimeriVax-DEN1	Preclinical (monkeys)	Guirakhoo *et al.*, 2000, 2001, 2002
	Dengue 2 (PUO218, wild-type) prM-E	YF 17D		ChimeriVax-DEN2	Preclinical (monkeys)	
	Dengue 3 (PaH881/88, wild-type) prM-E	YF 17D		ChimeriVax-DEN3	Preclinical (monkeys)	

	Dengue 4 (1228, wild-type) prM-E	YF 17D		ChimeriVax-DEN4	Preclinical (monkeys)	
	Dengue 1–4 (wild-type strains above)	YF 17D		ChimeriVax-DEN (tetravalent)	Preclinical (monkeys)	
Japanese encephalitis (JE)	JE (SA14-14-2, attenuated) prM-E	YF 17D	Acambis Inc.	ChimeriVax-JE	Phase II	Chambers *et al.*, 1999; Monath *et al.*, 2000, 2002b
West Nile	WN (NY 99, wild-type and attenuated) prM-E	YF 17D	Acambis Inc.	ChimeriVax-WN	Preclinical (mice, horses)	Arroyo *et al.*, 2001; Monath 2001
Tick-borne encephalitis (JE)	RSSE[b] (Sofjin) prM-E or C-prM-E	Dengue 4 (814669)	NIAID, NIH		Preclinical (mice)	Pletnev *et al.*, 1992, 1993
	Langat (TP21 wild-type and E5, attenuated)	Dengue 4 (814669)	NIAID, NIH		Preclinical (monkeys)	Pletnev *et al.*, 2000

[a] 30-nt deletion in the 3′ NCR (nt 172-143 from the 3′ terminus).
[b] Russian spring-summer encephalitis virus.

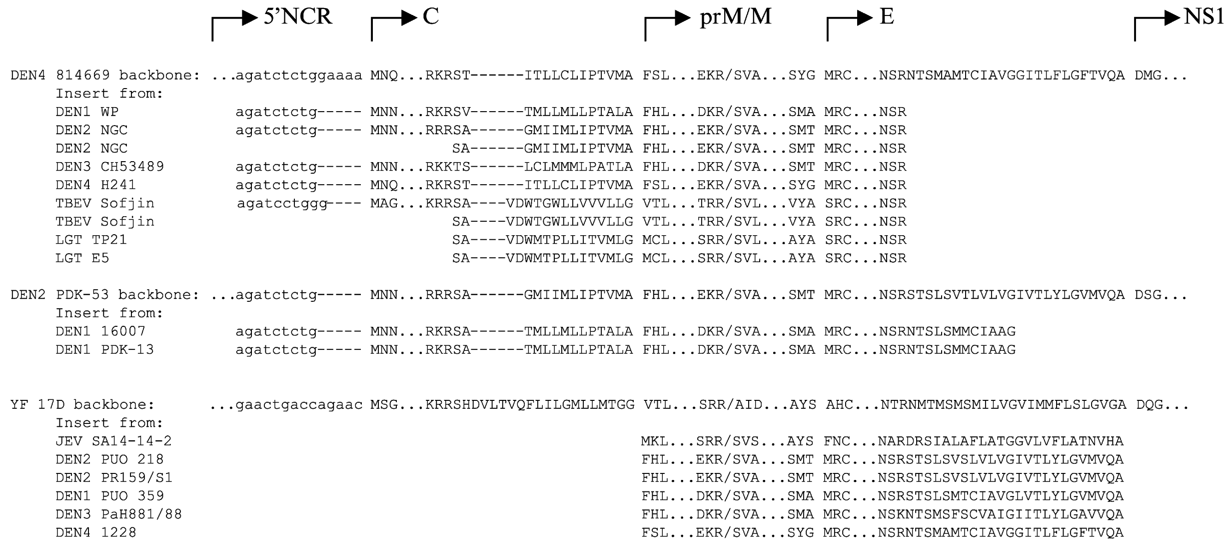

FIG 1. Chimeric flaviviruses that have been constructed using the genetic backbone derived from the full-length cDNA clone of the wild type DEN4 strain 814669, DEN2 PDK-53 candidate vaccine, or YF 17D vaccine. These chimeric viruses offer a novel strategy to accelerate development of live attenuated multivalent vaccines in the same genetic background against dengue, Japanese encephalitis, and tick-borne encephalitis. Shown are various inserts that contain the C-prM-E or only the prM-E structural protein genes from members of the flaviviruses used for substitution of the corresponding sequences in the full-length cDNA genome. Dashes are used for sequence alignment where appropriate. The genomic sequences upstream of the 5′NCR and downstream of the NS1 in each of the expression backbone that are not shown are represented by three dots. The three-dot symbol also denotes the intervening amino acid sequences that are not shown in various regions of the C, prM and E proteins. The slash symbol shows the furin cleavage site in the prM protein to generate the mature M. (Figure adapted from Fig. 2 of Kinney and Huang, 2001.)

TABLE II

Viremia and Antibody Response in Rhesus Monkeys following Immunization with Chimeric Intertypic DEN4/DEN1 and DEN4/DEN2 viruses, Singly or in Combination, or with Chimeric DEN4/LGT Virus and Protection against Challenge with Homologous Parental DEN1, DEN2, or LGT Virus

Immunizing virus (pfu)/dose	Viremic monkeys/ monkeys immunized	$PRNT_{50}$, against parental virus of immun. virus serotype	Challenge virus, (pfu)/dose	Viremic monkeys/ monkey challenged
DEN4, (3×10^5)[a]	3/4	135	DEN1, (3×10^5)	4/4
DEN4, (3×10^5)[a]	4/4	75	DEN2, (3×10^5)	4/4
DEN1, (3×10^5)	4/4	1076	DEN1, (3×10^5)	0/4
DEN2, (3×10^5)	4/4	1280	DEN2, (3×10^5)	0/4
DEN4/DEN1, (3×10^5)	2/4	320	DEN1, (3×10^5)	1/4
DEN4/DEN2, (3×10^5)	4/4	640	DEN2, (3×10^5)	0/4
DEN4/DEN1, (1.5×10^5) + DEN4/DEN2, (1.5×10^5)	3/4[b]	293[c]	DEN1, (3×10^5)	0/4
DEN4/DEN1, (1.5×10^5) + DEN4/DEN2, (1.5×10^5)	4/4[b]	452[d]	DEN2, (3×10^5)	0/4
DEN4/LGT, (10^3)	0/4	871	LGT, (10^5)	0/4
DEN4/LGT, (10^5)	0/4	372	LGT, (10^5)	0/4
DEN4/LGT, (10^7)	1/4	2334	LGT, (10^5)	0/4
LGT, (10^5)	4/4	3311	LGT, (10^5)	0/4
LGT, (10^7)	4/4	603	LGT, (10^5)	0/4
DEN4, (10^5)	4/4	1622	LGT, (10^5)	4/4

$PRNT_{50}$ shows the geometric mean titer determined for individual serum samples taken 56 days after immunization with DEN4, DEN1, DEN2, chimeric intertypic DEN4/DEN1 or DEN4/DEN2 virus grown in LLC-MK2 cells in the study of Bray *et al*. (1966). Serum samples were taken 42 days after immunization with DEN4, LGT or chimeric DEN4/LGT virus prepared in Vero cells (Pletnev *et al*., 2001).

[a] Cumulative data of two experiments.

[b] Viremia with DEN4/DEN2.

[c] Titer determined against DEN1.

C. Genetic Loci for Dengue Mouse Neurovirulence

Although the mouse is not a natural host for dengue viruses, each of the four human dengue virus serotypes can be adapted to grow in suckling mouse brain by serial IC passage (Hammon *et al*., 1960a, 1960b; Sabin and Schlesinger, 1945). This adaptation process selects mouse neurovirulent mutants that were also shown to be attenuated in humans (Hotta, 1952; Sabin, 1952; Schlesinger, 1977; Wisseman *et al*., 1963). The molecular basis for dengue virus neurovirulence in mice has been investigated. In the genetic background of non-neurovirulent DEN4 strain 814669, a chimeric virus containing the C-prM-E gene of a mouse-adapted, neurovirulent DEN4 strain H241 retained the neurovirulence phenotype for suckling mice. In contrast, a similar chimera containing the C-prM-E genes of the parental DEN H241 strain was not neurovirulent (Kawano *et al*., 1993) (Fig. 1). A comparison of the sequence in the C-prM-E region revealed a limited number of amino acid differences between the neurovirulent and non-neurovirulent DEN4 H241 strains. A Thr to Leu substitution at position 155 in E that abolished one of the two conserved glycosylation sites was largely responsible for acquisition of the dengue virus mouse neurovirulence phenotype. Mutational analysis of the DEN4/DEN2 chimeras containing the C-prM-E genes or only the prM-E genes of the prototype DEN2 NGC strain or its neurovirulent variant identified a Glu_{126}-to-Lys substitution in E as the dominant determinant of dengue virus type 2 mouse neurovirulence (Bray *et al*., 1998). That a single amino acid change in the E protein is responsible for dengue virus mouse neurovirulence was further demonstrated in studies with the DEN4/DEN3 (C-prM-E) chimeras expressing the DEN3 serotype antigenicity. The chimeric DEN4/DEN3 (C-prM-E) virus containing a substitution of Glu_{126}- to-Lys in E analogous to the neurovirulence mutation identified in DEN2 E was neurovirulent in mice, whereas the DEN4/DEN3 (C-prM-E) chimera containing the prototype DEN3 sequence was not (Chen *et al*., 1995). These observations serve to emphasize the need to maintain the genetic stability of live virus vaccines, despite the fact that dengue viruses are not significantly neurotropic in humans. The high mutation rate for adaptation upon passage of flaviviruses in cell cultures or in infected hosts could produce mutants having adverse effects.

D. Engineering Attenuating Mutations in the DEN4 Vector

Mutations have been introduced in several regions of the wild type DEN4 814669 genome to identify candidate live vaccines that exhibit reduced replication efficiency in cultured cells and in rhesus monkeys

(Cahour *et al.*, 1995; Lai *et al.*, 2001). Theoretically, deletion mutants are more stable and thus more suitable for use as vaccine strains than mutants containing point mutations. A DEN4 mutant with a 6 nucleotide (nt) deletion in the 5′ NCR (nt 82-87) formed small plaques on LLC-MK_2 cells and replicated inefficiently in these cells. Unlike wild type DEN4, this deletion mutant failed to produce plaques in C6/36 cells and did not replicate in *Ae. albopictus* or *Ae. aegypti* mosquitoes following intrathoracic inoculation (Cahour *et al.*, 1995).

A panel of viable DEN4 mutants with deletions ranging from 30 to 262 nt in the 3′ NCR have also been isolated and characterized (Men *et al.*, 1996). These DEN4 deletion mutants produced small plaques on C6/36 cells and grew to a lower titer in LLC-MK_2 cells, except for mutant 3′d 303-183 (lacking nt 303-183 from the 3′ terminus) which was not restricted for growth compared to the wild type DEN4. DEN4 mutant 3′d 303-183 and four other moderately restricted mutants (i.e., 3′d 333-183, 3′d 172-143, 3′d 172-113, and 3′d 384-183) were selected for evaluation of infectivity and immunogenicity in rhesus monkeys. An analysis of antibody response to infection provided evidence that several of these DEN4 deletion mutants were attenuated. Mutant 3′d 303-183 induced an antibody response (mean $PRNT_{50}$ titer of 370) equivalent to that stimulated by wild type DEN4. Four other mutants induced mean $PRNT_{50}$ titers ranging from 28 to 307. The immunogenicity of these 3′ NCR deletion mutants appeared to correlate with their replication efficiency in LLC-MK_2 cells. There was also evidence of deceased occurrence and duration of viremia for most of these mutants compared with the wild type DEN4 (Men *et al.*, 1996).

E. A DEN4 Mutant with a 30-nt Deletion in the 3′ NCR Is Highly Attenuated and Immunogenic in Humans

The DEN4 mutant with a 30-nt deletion in the 3′ NCR (nt 172-143 from the 3′ terminus) exhibited reduced replication capacity in rhesus monkeys and elicited a moderate level of neutralizing antibodies and might prove to be an interesting vaccine candidate (Men *et al.*, 1996). Starting from the cDNA clone with the 30 nt deletion, a DEN4 candidate vaccine was produced in certified Vero cells to evaluate the safety and immunogenicity in a Phase I clinical trial (Durbin *et al.*, 2001). A single dose of 10^5 pfu of the DEN4 candidate vaccine was administered SC to 20 dengue virus seronegative volunteers. The vaccine was well tolerated and induced a satisfactory level of serum neutralizing antibodies, with a mean $PRNT_{50}$ titer of 580. A mild macular rash developed in 10 volunteers and a transient elevation in serum ALT

level was noted in five volunteers. This was regarded as a low level of reactogenicity and was comparable to that seen in studies of attenuated dengue virus vaccines (Edelman *et al.*, 1994; Kanesa-thasan *et al.*, 2001; Vaughn *et al.*, 1996). The DEN4 genome with the 30-nt deletion in the 3′ NCR is an attractive vector for the construction of intertypic dengue chimeras expressing the antigenic regions of DEN1, DEN2, DEN3.

IV. Chimeric Flaviviruses against TBE Using DEN4 as the Backbone

A. *Chimeric DEN4/TBEV Viruses*

The chimeric flavivirus strategy using the wild type DEN4 cDNA backbone has been extended to construct vaccine candidates against the distantly related TBEV (Pletnev *et al.*, 1992). Two chimeric DEN4/TBEV (C-prM-E) and DEN4/TBEV (prM-E) viruses, which contained the C-prM-E or only the prM-E genes from the highly virulent TBEV Sofjin strain with the remaining sequences from DEN4, were recovered from LLC-MK_2 cells (Fig. 1). The recovery of the DEN4/TBEV (C-prM-E) chimera was slow compared to that of the DEN4/TBEV (prM-E) chimera, reminiscent of the construction of DEN4/DEN2 chimeras containing the DEN2 (C-prM-E) or (prM-E) genes. Host cell restriction and the neurovirulence in mice were used to characterize the growth phenotype of the chimeric DEN4/TBEV (prM-E) virus, hereafter called DEN4/TBEV, derived from flaviviruses that are transmitted by different arthropod vectors. The DEN4/TBEV chimera produced larger plaques and replicated more efficiently in simian LLC-MK_2 cells than the parental DEN4. In contrast, the DEN4/TBEV chimera formed smaller plaques and grew to a titer 100-fold lower than DEN4 in mosquito C6/36 cells. In the non-neurovirulent background of DEN4, the DEN4/TBEV chimera caused fatal encephalitis in suckling or adult mice inoculated IC with 10^2 or 10^3 pfu, respectively. The DEN4/TBEV chimera retained the neurovirulence of TBEV, from which its prM and E genes were derived. Thus, the TBEV mouse neurovirulence loci mapped within the prM-E genes. The DEN4/TBEV mouse neurovirulence demonstrated by IC inoculation was reduced by the substitution of a single amino acid in the TBEV prM, or E or DEN4 NS1 gene of the chimera (Pletnev *et al.*, 1993). Each of these amino acid substitutions caused a restriction in replication of the chimera in LLC-MK_2 or C6/36 cells.

Chimerization of genes from two distantly related flaviviruses, such as the DEN4 and TBEV, might create functional incompatibilities resulting in reduced viral replication capacity *in vitro* or *in vivo*. This possibility could explain the observation that the DEN4/TBEV chimera lacked the peripheral invasiveness of TBEV (Pletnev *et al.*, 1992). TBEV is highly virulent with a LD_{50} of 14 pfu in adult mice by a peripheral route. In contrast, all adult mice survived after inoculation with 10^3 pfu of the DEN4/TBEV chimera by the IP or intradermal route. Nevertheless, mice peripherally inoculated with the chimera induced complete resistance to fatal encephalitis caused by lethal challenge with TBEV. Similarly, mice inoculated IP with 10^4 pfu of the DEN4/TBEV chimeras containing the attenuating mutations in the TBEV prM or E, or DEN4 NS1 gene were resistant to lethal IC challenge with the neurovirulent, parental DEN4/TBEV chimera (Pletnev *et al.*, 1992, 1993). Thus, the DEN4/TBEV chimera contains the major protective antigens, but lacks the peripheral neuroinvasiveness of TBEV. These observations establish the basis of the chimeric virus strategy for developing a live-attenuated vaccine against the highly virulent TBEV.

B. Chimeric DEN4/LGT(TP21) and DEN4/LGT(E5) Viruses

Langat (LGT) prototype TP21 strain was identical to a candidate vaccine evaluated earlier in Russia for prevention of TBEV (Mandl *et al.*, 1991; Smorodintsev, 1986). Studies in the former Soviet Union to evaluate this vaccine were halted because of serious adverse events (vaccine-associated encephalitis). LGT TP21 and its mutant derivative, E5, attenuated by serial passage in embryonic chicken eggs, remained neurovirulent in mice, although less so than TBEV (Nathanson *et al.*, 1968; Price and Thind, 1973; Thind and Price, 1966). In an attempt to remove the residual mouse neurovirulence of LGT TP21 and LGT E5, chimeric viruses have been constructed between the prM-E genes of the LGT TP21 or E5 strain and the remaining sequence from the DEN4 814669 strain (Pletnev and Men, 1998). Viable chimeric DEN4/LGT(TP21) and DEN4/LGT(E5) viruses were recovered from C6/36 cells (Fig. 1). These chimeras were at least 5000 times less neurovirulent than their parental LGT viruses in suckling mice and did not exhibit neuroinvasiveness following IP inoculation of outbred Swiss mice. Mice immunized IP with 10^5 pfu of either of these chimeras developed a mean $PRNT_{50}$ titer of approximately 300 at 21 days after infection. Mice immunized IP with as little as 10 pfu of each of these chimeras responded with a $PRNT_{50}$ titer of approximately 60 and were

resistant to lethal IP challenge with 1000 LD_{50} of wild type LGT TP21. Thus, evidence indicates that the protective immunity is induced following viral replication *in vivo*, but not the result of immunization with the preformed antigens.

C. Mice Immunized with Chimeric DEN4/LGT(TP21) or DEN4/LGT(E5) Are Protected against Lethal TBEV Challenge

Following repeated passages in Vero cells, both DEN4/LGT(TP21) and DEN4/LGT(E5) grew to a titer greater than 3×10^6 pfu/mL. There were limited amino acid differences in the prM-E region of these chimeras following adaption for growth in Vero cells (Pletnev *et al.*, 2000). The Vero cell–adapted chimeric viruses retained the avirulent phenotype for 3-week-old outbred Swiss mice as well as immunodeficient SCID mice. Inbred CBA mice immunized with a single dose of 600 pfu of either chimeric vaccine were only partially protected against lethal IP challenge by heterologous TBEV strain Absettarov. Complete protection was achieved after two inoculations with the DEN4/LGT virus. Similarly, complete protection was observed when inbred BALB/c mice were inoculated with the candidate vaccine multiple times. Protection of outbred Swiss mice against TBEV strain Sofjin by inoculation with 10^5 pfu of the chimeric vaccine was also carried out. Mice immunized twice with either DEN4/LGT(TP21) or DEN4/LGT(E5) chimera developed LGT neutralizing antibodies with a mean $PRNT_{50}$ titer of 452 or 96, respectively. The DEN4/LGT(TP21) chimera was able to confer complete protection after two inoculations, whereas only 67% of mice inoculated with two doses of DEN4/LGT(E5) survived the heterologous TBEV challenge. There was a close correlation between the level of neutralizing antibodies induced in mice following immunization with the chimeras and protection against TBEV. Although the parental LGT viruses are more immunogenic and protective than their DEN4 chimeric derivatives, the chimeric viruses offer greater safety and still achieve an equivalent protective efficacy with a two-dose regimen (Pletnev *et al.*, 2000).

D. Immunogenicity and Protective Efficacy of Chimeric DEN4/LGT in Monkeys

The chimeric DEN4/LGT(TP21) vaccine prepared in Vero cells has been evaluated for attenuation, immunogenicity and protective efficacy in rhesus monkeys (Pletnev *et al.*, 2001). Viremia was detected only in one of 12 monkeys in groups of four immunized SC with 10^3,

10^5, or 10^7 pfu of the chimeric DEN4/LGT(TP21) virus (Table II). In contrast, viremia was detected in each of the four monkeys inoculated SC with 10^5 or 10^7 pfu of LGT TP21 or with 10^5 pfu of DEN4 grown in Vero cells. Monkeys in the groups immunized with DEN4/LGT(TP21) developed mean $PRNT_{50}$ titers of 372–2344 against LGT TP21 or mean $PRNT_{50}$ titers of 320–640 against heterologous TBEV. These antibodies were able to passively protect mice against IP challenge with 100 LD_{50} of the highly virulent TBEV Sofjin strain. Six weeks after immunization, these monkeys were challenged with 10^5 pfu of LGT TP21 to assess the protective efficacy. None of the 12 monkeys immunized with the chimeric virus vaccine, nor any of the eight monkeys immunized with LGT TP21 became viremic after challenge. Monkeys in the group previously immunized with DEN4 became viremic following LGT P21 challenge.

The non-hematophagous mosquitoes of the genus *Toxorrhynchites* are a sensitive host for determining the infectivity of dengue viruses (Rosen, 1981). Intrathoracic inoculation of *T. splendens* has been investigated to address the vector transmissibility of the chimeric DEN4/LGT (TP21) vaccine (Pletnev *et al.*, 2001). Viral infection was not detected in this mosquito species inoculated with $10^{4.6}$ pfu of LGT TP21 or with $10^{4.3}$ pfu of chimeric DEN4/LGT(TP21). In contrast, DEN4 replicated efficiently in these mosquitoes with a 50% infectious dose of $10^{2.8}$ pfu. The failure of the DEN4/LGT(TP21) virus to infect *T. splendens* and a very low level of viremia response in monkeys suggest that this chimera is unlikely to be transmitted by mosquitoes. These studies demonstrate that the chimeric DEN4/LGT(TP21) vaccine candidate has a favorable safety profile. Chimerization between wild type DEN4 and its distantly related tick-borne flavivirus LGT completely ablated neuroinvasiveness of LGT in mice. The degree of attenuation characteristics as well as its satisfactory immunogenicity and protective efficacy of the sequence-unmodified chimeric DEN4/LGT virus in monkeys makes it a promising vaccine strategy against TBEV.

V. Chimeric Flaviviruses against Dengue Using Attenuated DEN2 PDK-53 as the Backbone

A. Molecular Basis for Attenuation of DEN2 PDK-53 Virus

A tetravalent dengue virus vaccine has been prepared by Aventis-Pasteur and consists of the DEN1 PDK-13, DEN2 PDK-53, DEN3 PGMK-30/FRhL-3, and DEN4 PDK-48 viruses attenuated by empirical

serial passage of their respective DEN116007, DEN2 16681, DEN3 16562, and DEN4 1036 wild type strains in primary dog kidney or monkey kidney cells (Bhamarapravati and Yoksan, 1997). Among these candidate vaccines, DEN2 PDK-53 is the most infectious for humans with 50% minimum infectious dose (MID_{50}) of 5 pfu (Bhamarapravati and Yoksan, 1997). DEN2 PDK-53 has been evaluated in various phase I clinical trials as a monovalent vaccine (Bhamarapravati *et al.*, 1987; Vaughn, 1996) and in multivalent combinations with DEN1 or DEN4, with DEN1 and DEN4, or with DEN1, DEN3 and DEN4 vaccine candidates (Bhamarapravati and Yoksan, 1989; Bhamarapravati and Sutee, 2000). Immunization with the DEN2 PDK-53 vaccine at 10^4 pfu/dose elicited mean $PRNT_{50}$ titers of 215–230 on day 60 in adult human volunteers with acceptable reactogenicity. DEN2 PDK-53 exhibited small plaque size morphology, temperature sensitivity in LLC-MK_2 cells, inefficient replication in C6/36 cells, and decreased neurovirulence in suckling mice compared to the parental DEN2 strain.

Full-length cDNA clones from the DEN2 PDK-53 virus and the parental DEN2 strain 16681 have been constructed (Butrapet *et al.*, 2000). Mutational analysis identified three mutations: i) a nucleotide substitution at position 57 in the 5′ NCR; ii) a Gly-Asp substitution at NS1 position 53; and iii) a Glu-Val substitution at NS3 position 250 in the DEN2 PDK-53 genome, as responsible for the attenuation characteristics *in vitro* and reduced mouse neurovirulence *in vivo*. Restoration of at least two of the three loci was required to display the wild type characteristics of DEN2 16681 *in vitro*. Because all three mutations are located outside the structural protein gene region, the DEN2 PDK-53 cDNA is a unique vector for construction of attenuated intertypic dengue viruses expressing the structural genes of other dengue virus serotypes (Butrapet *et al.*, 2000) and intratypic YF/DEN2 chimeras (Guirakhoo *et al.*, 2000).

B. Chimeric DEN2/DEN1 Viruses

Among the four dengue virus serotype strains in the tetravalent vaccine of Aventis Pasteur, DEN1 PDK-13 is the least infectious and requires 10,000 pfu to achieve MID_{50} in humans (Bhamarapravati and Yoksan, 1997). The low infectivity suggests that the DEN1 PDK-13 candidate vaccine might be over-attenuated. There are eight amino acid differences between the DEN1 PDK13 and its parental DEN1 16007 strains (Huang *et al.*, 2000). Among these, five are present in the E protein, two in NS3 and one in NS4A. The chimeric DEN2/DEN1 (PDK-13) and DEN2/DEN1 (16007) viruses that contain the

C-prM-E genes of DEN1 PDK-13 vaccine or its parental DEN1 16007 strain in the nonstructural protein gene background of DEN2 PDK-53 or its parental DEN2 16681 virus have been constructed to assess their attenuation characteristics (Huang *et al.*, 2000). The chimeric DEN2 (16681)/DEN1 (16007) virus grew less efficiently than either parental virus in C6/36 cells, indicating an effect of chimerization between heterologous dengue virus genes. The DEN2 (PDK-53)/DEN1 (16007) and DEN2 (PDK-53)/DEN1 (PDK-13) chimeras retained the DEN2 PDK-53 attenuation markers of small plaque morphology, temperature sensitivity in LLC-MK_2 cells and inefficient replication in C6/36 cells, as compared to wild type DEN1 16007. The immunogenicity of the DEN1 16007, DEN1 PDK-13 and their derived chimeras was analyzed in a mouse model. Immunization of 3-week-old outbred ICR mice with 10^4 pfu of the chimeric DEN2 (PDK-53)/DEN1 (16007) virus elicited $PRNT_{50}$ titers of 2560–10240 following a booster inoculation. Mice similarly inoculated with parental DEN1 16007 had a comparable $PRNT_{50}$ titer of 2560. However, the DEN2 (PDK-53)/DEN1 (PDK-13) chimera that contained the C-prM-E genes of the attenuated DEN1 PDK-13 virus elicited much reduced $PRNT_{50}$ titers of 80–160 under the same conditions. The five amino acid changes identified in E, therefore, contributed to the reduced growth profile of the DEN2 (PDK-53)/DEN1 (PDK-13) virus in LLC-MK_2 and C6/36 cells and low immunogenicity in mice, consistent with low infectivity of the DEN1 PDK-13 vaccine in humans. The highly immunogenic DEN2 (PDK-53)/DEN1 (16007) chimera containing the C-prM-E genes of wild type DEN1 16007 could be considered as a promising substitute (Huang *et al.*, 2000). These studies have established the basis for construction of intertypic dengue viruses to express the C-PrM-E genes of the wild type DEN3 and DEN4 strains in the same genetic background of attenuated DEN2 PDK-53. These intertypic dengue viruses are novel vaccine candidates for further analysis of attenuation, immunogenicity, and protective efficacy in primates.

VI. Chimeric Flaviviruses Using YF 17D Vaccine Strain as the Backbone

The live-attenuated YF 17D vaccine developed in 1936 has proved to be a safe and effective live attenuated vaccine, which has been used in over 400 million persons (Monath, 1999). It is delivered as a single subcutaneous inoculation and induces neutralizing antibodies (the

mediator of protection) in 90% of vaccinees within 10 days and 99% within 30 days after inoculation. The 50% effective dose is only 50 pfu. Immunity is very durable and probably lifelong. These characteristics make it an ideal vector for heterologous genes encoding protective antigens of other flaviviruses. Chimeric vaccines based on YF 17D are at various stages of development against JE, DEN, and WN viruses. The technology was pioneered by T. Chambers at St. Louis University (Chambers *et al.*, 1997, 1999), and subsequently developed by Acambis Inc. Aventis Pasteur has licensed the technology from Acambis for DEN vaccine development. Funding has also been provided from the World Health Organization and the National Institute of Allergy and Infectious Diseases (National Institutes of Health).

An important aspect of the technology is that preclinical and clinical activities of chimeric YF vaccines can be measured against control subjects who receive the commercial YF 17D vaccine. Comparability of experimental and licensed products provides assurance that the new vaccines will be safe and effective in humans. Because YF 17D virus retains the ability to kill mice of all ages after IC inoculation, and to cause subclinical encephalitis after IC inoculation of monkeys, comparisons of the biologic characteristics of YF 17D and chimeric constructs are used to assess the safety profile of the chimeras.

A. Chimeric YF/JE Vaccine (ChimeriVax-JE)

The vaccine candidate ChimeriVax-JE is a live, attenuated, genetically engineered virus prepared by replacing the prM-E genes of YF 17D vaccine virus with the corresponding genes of JE virus (Chambers *et al.*, 1999). The prM and E proteins of JE virus contain the critical antigens conferring protective humoral and cellular immunity, as shown by many previous studies with recombinant subunits, poxvirus vectors, and DNA vaccines (Konishi *et al.*, 1998; Mason *et al.*, 1991).

The prM-E genes in the chimeric vaccine were derived from the JE SA14-14-2 strain, a live-attenuated vaccine strain licensed for use in China. The biologic properties and molecular characterization of this strain are well documented and it is a safe and effective human vaccine. The genetic rearrangement was accomplished by standard cloning techniques, using two bacterial plasmids containing cDNA copies of the prM-E genes of JE SA14-14-2 virus and the remaining genes of YF 17D (Chambers *et al.*, 1999).

Extensive preclinical studies have been undertaken of the safety profile, immunogenicity, and protective activity of ChimeriVax-JE. Studies in mice (Guirakhoo *et al.*, 1999) and monkeys (Monath *et al.*,

1999, 2000) showed that a single dose of ChimeriVax-JE was highly immunogenic and protected the animals against lethal (IC) JE virus challenge. ChimeriVax-JE virus was fully attenuated for weaned mice inoculated by the IC route, whereas commercial YF 17D vaccine (YF-VAX®) caused lethal encephalitis with an LD_{50} of 1.67 $\log_{10}$ pfu. To evaluate immunogenicity and protection, groups of four rhesus monkeys were inoculated by the SC route with 2.0, 3.0, 4.0, or 5.0 $\log_{10}$ PFU of ChimeriVax-JE virus. All 16 monkeys developed low-level viremia (mean peak 1.7–2.1 $\log_{10}$ pfu/mL; mean duration 1.8–2.3 days), similar to those induced by YF 17D. Neutralizing antibodies appeared between days 6 and 10, and by day 30 responses were similar across dose groups, with $PRNT_{50}$ titers between 320 and 2560. All immunized monkeys and sham-immunized controls were challenged by the IC route with 5.2 $\log_{10}$ pfu of wild-type JE virus. None of the immunized monkeys developed viremia or illness and had mild residual brain lesions 30 days after challenge, while sham-inoculated controls developed viremia, lethal encephalitis, and severe histopathologic lesions. Challenge by the IC route is a severe test of immunity, since natural infection occurs by intradermal inoculation during mosquito feeding and the virus must undergo extraneural replication and cross the blood-brain barrier.

The attenuated phenotype of ChimeriVax-JE is conferred in part by the YF 17D nonstructural genes and in part by the attenuated prM-E sequence from the SA14-14-2 vaccine strain. This was demonstrated by Chambers *et al.* (1999), using a chimeric YF/JE construct containing the donor prM-E genes of wild-type JE Nakayama virus. The latter was significantly more neurovirulent than the YF/JE SA14-14-2 virus but less virulent than JE-Nakamaya virus.

Further dissection of the molecular basis for attenuation was accomplished by Arroyo *et al.* (2001a). Ten amino acid differences exist between the E proteins of ChimeriVax-JE and the wild-type JE (Nakayama) virus. Four of these residues, (E_{107}, E_{138}, E_{176}, and E_{279}), are suspected to play a critical role in neurovirulence. Site-directed mutagenesis of the chimeric YF/JE SA14-14-2 was undertaken to revert one or multiple SA14-14-2-specific determinants to the wild-type residues. Neurovirulence of the revertants was then assessed by IC inoculation of adult outbred mice. This analysis showed that reversion of three or four amino acids was required to restore the mouse neurovirulence typical of the wild-type JE virus. This finding, as well as the stability of the SA14-14-2 specific residues during sequential passage (Guirakhoo *et al.*, 1999), indicated that the risk of reversion to virulence was exceedingly remote. An additional safety

feature was the finding that ChimeriVax-JE vaccine was incapable of infecting *Culex* and *Aedes* mosquitoes by oral feeding and had markedly restricted replication after direct intrathoracic inoculation (Bhatt *et al.*, 2001).

The Lys-Met substitution at E279 of YF/JESA14-14-2 virus was subsequently studied in more detail using more sensitive methods for revealing subtle increases in neurovirulence (Monath *et al.*, 2002a). This mutation is located in the hinge region of the E protein responsible for a pH-dependent conformational change during internalization. Single-site reversion to the wild-type Lys residue significantly increased neurovirulence determined by LD_{50} and survival distribution in suckling mice and by histopathology in rhesus monkeys inoculated IC. Although these results seem to contradict the conclusion that a single site reversion would be detrimental to the safety profile of the vaccine, it should be emphasized that virulence of the Met-Lys revertant was still markedly less than that of YF 17D. Interestingly, the revertant virus was restricted with respect to extraneural replication in monkeys, as viremia and antibody levels (markers of viscerotropism) were significantly reduced compared to virus containing methionine at position 279 of the E protein. These results are consistent with the observation that vaccines developed by neuroadaptation (mouse-brain passage) of dengue and YF (French neurotropic strain) have increased neurovirulence for mice but reduced viscerotropism for humans.

A randomized, double-blind, outpatient phase I study of ChimeriVax-JE was conducted to compare its safety and immunogenicity with that of YF 17D (Monath *et al.*, 2002b). This study also addressed the theoretical concern that previous YF immunization could interfere with a chimeric vaccine, due to anti-vector immunity conferred by anti-NS1 antibody or cell-mediated responses against NS3 or other YF proteins in the construct. Six YF immune and six non-immune adults were randomized to receive a single SC inoculation of ChimeriVax-JE (5 $\log_{10}$ PFU), ChimeriVaxTM-JE (4 $\log_{10}$ PFU), or YF-VAX (5 $\log_{10}$ PFU). Mild, transient injection site reactions and flu-like symptoms were noted in all treatment groups, with no significant difference between the groups. Nearly all subjects inoculated with ChimeriVax-JE at both dose levels developed a transient, low-level viremia that was similar in magnitude and duration to that following YF-VAX. Neutralizing antibody seroconversion rates to JE were 100% in the high and low dose groups in both naïve and YF-immune subjects. Mean neutralizing antibody responses were higher in the ChimeriVax-JE high dose groups (naïve subjects $PRNT_{50}$ 254; YF immune subjects $PRNT_{50}$ 327) than in the low dose groups (naïve subjects $PRNT_{50}$ 128; YF

immune subjects $PRNT_{50}$ 270). Previous yellow fever immunity either did not change or increased the neutralizing antibody response to the chimeric vaccine, probably due to memory responses to shared antigenic determinants in the E glycoprotein or carrier priming mediated by preexisting immunity to nonstructural proteins.

In a follow-up study, 10 adults who had been inoculated 9 months earlier with ChimeriVax-JE vaccine and 10 control subjects were challenged with non-infectious JE virus antigen, representing a surrogate for exposure to live virus transmitted by mosquitoes under conditions of natural exposure. The challenge virus used was a formalin-inactivated JE vaccine (JE-VAX). Six of 10 subjects who had previously received ChimeriVax-JE responded with a rapid increase in neutralizing antibodies between challenge and Day 7. Of the four subjects who had no increase antibodies during this interval, three already had protective antibody levels before challenge. Eight of 10 subjects responded with a significant increase in antibodies by 14 days after challenge. Mean antibody levels on Day 7 were approximately 20-fold higher and on Day 14 100-fold higher than pre-inoculation levels. In contrast, none of the control subjects that received JE-VAX developed antibodies by Day 7. Differences in mean antibody levels between the treatment groups were highly significant on all study days. These results demonstrate that strong immunologic memory is induced by the ChimeriVax-JE vaccine. Vaccinated individuals would be expected to have a rapid anamnestic response if exposed to wild-type JE virus.

A phase II clinical trial has recently been completed to investigate the safety and immunogenicity of ChimeriVax-JE vaccine in subjects who received one or two inoculations (30 days apart) across a wide dose range (1.8–5.8 $\log_{10}$ pfu) (Monath *et al.*, 2003). Treatment groups included volunteers who received YF 17D 30 days before or after inoculation of ChimeriVax-JE. This was a randomized, double blind, placebo-controlled, outpatient study in 99 subjects. Eleven subjects were assigned to each of nine groups on a random basis (Table III). Sample size was based on the responses observed in a previous clinical trial of ChimeriVax-JE (Monath *et al.*, 2002b). A sample size of 10 subjects per group provided 90% power to establish a dose response among groups 1 through 5 (Table III) in a logistic regression model for the proportion seroconverting at Day 30 at significance level 0.05. The study was conducted in healthy male and female adults 18–59 years old at a single center in the United States. Subjects were excluded who had significant medical conditions; contraindications to YF 17D (YF-VAX, Aventis Pasteur) vaccine (pregnancy, immunosuppression, egg hypersensitivity); positive test results for HBsAg or for hepatitis C or HIV

antibody; pregnancy or lactation (females); or a history of vaccination against YF or JE.

The objectives of the study were to:

1. Assess the safety and tolerability of ChimeriVax-JE compared with YF-VAX and placebo using laboratory tests (including quantitative viremia measurements), body temperature, and the incidence of adverse events;
2. Characterize the antibody response following administration of ChimeriVax-JE vaccine by:
    a. Determining the proportion of subjects seroconverting (by neutralization test) after administration of tenfold graded doses vaccine between 1.8 and 5.8 $\log_{10}$ pfu;
    b. Comparing the immune response to a single dose of ChimeriVax-JE with the immune response to two doses separated by 30 days;
    c. Determining the effect on antibody levels of sequential vaccination with ChimeriVax-JE followed by YF-VAX and YF-VAX followed by ChimeriVax-JE administered at a 30-day interval.

The vaccines were well tolerated. There were no serious adverse events related to study medication. The majority of subjects in all groups reported one or more treatment-emergent adverse events. The symptom pyrexia was considered possibly or definitely related to ChimeriVax-JE vaccine in five subjects one each in groups 1, 3, 4, 5, and 9) and at least possibly related to YF-VAX in two subjects. Adverse events were more frequent after the first than after the second dose. There were no differences in the incidence of adverse events across active vaccine and placebo treatment groups after the first dose ($P = 0.7874$, Fisher's exact test) or second dose ($P = 0.7626$). The most frequently reported adverse events were injection site erythema and pain, fatigue, myalgia, headache, and diarrhea. Whereas injection site reactions were attributable to the study vaccines, systemic adverse events were less clearly associated.

Viremia in ChimeriVax-JE subjects was characterized by short duration and low titer. Fifty to 100% of subjects in all ChimeriVax-JE treatment groups and 64% of subjects inoculated with YF-VAX developed detectable viremia on one or more days after inoculation. The viremia following YF-VAX occurred quite uniformly between days 4 and 6, whereas ChimeriVax-JE subjects had a more variable pattern, with some subjects having early, late, or intermittent viremias. Viremia summary data are shown in Table III.

TABLE III

PHASE 2 CLINICAL TRIAL OF CHIMERIVAX-JE: DESIGN AND VIREMIA DATA

Group number (n = 11 per group)[a]	Treatment		Viremia mean peak[c] (PFU/mL)	Viremia mean duration (days)[c]
	Day 0	Day 30		
1	CV-JE[b] (5.8 $\log_{10}$ pfu)	CV-JE (5.8 $\log_{10}$ pfu)	7.0	0.9
2	CV-JE (4.8 $\log_{10}$ pfu)	CV-JE (4.8 $\log_{10}$ pfu)	13.0[d]	1.6[d]
3	CV-JE (3.8 $\log_{10}$ pfu)	CV-JE (3.8 $\log_{10}$ pfu)	16.4	1.4
4	CV-JE (2.8 $\log_{10}$ pfu)	CV-JE (2.8 $\log_{10}$ pfu)	40.9	2.7
5	CV-JE (1.8 $\log_{10}$ pfu)	CV-JE (1.8 $\log_{10}$ pfu)	18.2	2.2
6	CV-JE (4.8 $\log_{10}$ pfu)	YF-VAX	[d]	[d]
7	YF-VAX	CV-JE (4.8 $\log_{10}$ pfu)	21.8	1.2
8	Placebo	CV-JE (4.8 $\log_{10}$ pfu)	0	0
9	CV-JE (4.8 $\log_{10}$ pfu)	Placebo	[d]	[d]

[a] Subjects were assigned to groups on a random basis.
[b] ChimeriVax-JE.
[c] Following first dose.
[d] All ChimeriVax-JE 4.8 $\log_{10}$ pfu dose groups combined to calculate mean values.

Eighty-two (94.3%) of 87 subjects who underwent primary immunization with a single inoculation of ChimeriVax-JE at all dose levels (groups 1–6, 8, 9) seroconverted to JE by neutralization test within 30 days. Seroconversion rates varied between 82% and 100% across the range of doses from 1.8 to 5.8 $\log_{10}$ pfu without relationship to dose. The magnitude of the neutralizing antibody response to ChimeriVax-JE was similar across dose groups. By 30 days after primary immunization, mean LNI values ranged from 1.38 to 2.02. No statistical differences in mean antibody titers were found across dose groups ($P = 0.7070$, ANOVA). Antibody titers increased rapidly over 2–3 weeks after primary inoculation, appeared to peak around day 30, did not increase after boosting, and in fact tended to decrease slightly by day 60.

The neutralizing antibody response to ChimeriVax-JE was not influenced by vaccination against YF performed 30 days previously. All 11 YF-naïve subjects in groups 6 and 10 (91%) of 11 YF-immune subjects in group 7 developed JE antibodies after inoculation of ChimeriVax-JE ($P = 1.000$, Fisher's exact test). The mean LNI to ChimeriVax-JE 30 days after inoculation was not significantly different in YF-naïve (1.92) and YF-immune subjects (1.42; $P = 0.0935$, t test). Subjects in group 6 received ChimeriVax-JE followed by YF-VAX® 30 days later. There was a suggestion that prior inoculation of ChimeriVax-JE diminished the serological response to YF 17D. Sixty-four percent of ChimeriVax-JE-immune subjects (group 6) seroconverted to YF, compared to 91% of ChimeriVax-JE-naïve subjects (group 7); the difference was, however, not statistically significant ($P = 0.3108$, Fisher's exact test). The mean LNI to YF 30 days after inoculation was lower in ChimeriVax-JE–immune subjects (group 6, 1.59) than in ChimeriVax-JE–naïve subjects (group 7, 2.29), but again the difference was not statistically significant ($P = 0.2256$, t test).

It was concluded from these results that ChimeriVax-JE has a safety profile and viremia pattern consistent with those of YF 17D vaccine. ChimeriVax-JE rapidly elicits high titers of neutralizing antibodies after a single inoculation at very low doses, an advantage over existing inactivated vaccines that require multiple doses. Additional clinical trials are planned to elucidate the safety and efficacy profile of this vaccine.

B. Chimeric YF/DEN Vaccine (ChimeriVax-DEN)

Several groups have independently reported the successful construction of chimeric YF/DEN2 viruses (Caufour *et al.*, 2001; Chambers *et al.*, 2003; Guirakhoo *et al.*, 2000; van der Most *et al.*, 2000). Guirakoo

et al. (2000) and Chambers *et al*. (2003) used donor prM-E genes from a low-passage Thai virus strain, whereas the other authors used DEN2 prototype New Guinea C virus (Caufour *et al*., 2001) or the Americas I genotype (PR159) (van der Most *et al*., 2000). It was hypothesized that wild-type donor genes would be suitable for construction of a vaccine candidate because the empirically derived PDK53 vaccine strain that was attenuated for humans contained no mutations in the E gene. All chimeric YF/DEN2 viruses were less neurovirulent than YF 17D after IC inoculation of mice and were immunogenic in mice. The YF/DEN-4 construct of Chambers *et al*. (2003) used a mouse-neuroadapted DEN-4 gene donor strain and the resulting chimera was neurovirulent. The YF/DEN constructs immunized mice and protected against intracerebral challenge with neurovirulent dengue strains (Chambers *et al*., 2003). Guirakhoo *et al*. (2000) showed that the YF/DEN2 vaccine inoculated SC over a wide dose range, caused low-titer viremia in rhesus monkeys compared to wild-type virus, elicited strong neutralizing antibody responses, and protected monkeys against wild-type DEN2 challenge.

The first recombinant tetravalent DEN vaccine was developed by Acambis (Guirakhoo *et al*., 2001). Following construction of the YF/DEN2 virus, chimeric vaccine candidates against the remaining three DEN serotypes were developed. The origin of the donor wild-type strains is shown in Table I. Different methods for genetic constructions were used, including the standard two-plasmid system, and *in vitro* ligation of an overlap extension PCR amplicon (YF/DEN1) or multiple DNA fragments (YF-DEN3). All YF/DEN1-4 chimeric viruses grew to high titers (approximately 7.5 $\log_{10}$ pfu/mL) in Vero cells, and were immunogenic in monkeys inoculated with monovalent or tetravalent formulations comprised of a mixture of equal (approximately 4 $\log_{10}$ pfu) doses of each monovalent chimera. Viremia in monkeys was similar to that induced by YF 17D and significantly lower than that induced by the wild-type parental dengue strains. All monkeys inoculated with the tetravalent vaccine developed neutralizing antibodies to all four serotypes (Table IV). Neutralizing antibody titers were similar to those induced by the monovalent vaccines. Monkeys were boosted with a second dose of tetravalent vaccine 6 months later. All had a marked anamnestic response, and developed high-titer antibody responses against all four serotypes. Monkeys previously vaccinated against YF 17D tended to have higher DEN antibody responses to the tetravalent vaccine compared to YF-naive animals. Similarly, monkeys given chimeric tetravalent vaccine 6 months before YF-VAX had similar

TABLE IV

TETRAVALENT CHIMERIC YELLOW FEVER 17D/DENGUE (CHIMERIVAX-DEN) VACCINE: NEUTRALIZING ANTIBODY RESPONSES IN RHESUS MONKEYS

					Geometric Mean PRNT50			
Experiment no.	Vaccine	Dose formulation	Inoculation	Day	DEN1	DEN2	DEN3	DEN4
1	ChimeriVax-DEN (tetravalent)	~4.0 $\log_{10}$ pfu of each serotype	Primary	0	<10	<10	<10	<10
				30	160	>1015	80	32
				79	101	1015	34	80
		~5.0 $\log_{10}$ pfu of each serotype	Boost	180	142	905	127	71
				210	640	1810	452	359
2	ChimeriVax-DEN (tetravalent)	DEN1 4.5; DEN2 3.0; DEN3 3.6; DEN4 4.4 $\log_{10}$ pfu	Primary	0	<10	<10	<10	<10
				30	359	403	254	1437

From Guirakhoo *et al.*, 2001, 2002.

anti-YF neutralizing antibody responses as naïve animals. These results indicated that there were no significant interactions between YF 17D vaccine and ChimeriVax, supporting the conclusion reached in a clinical trial of ChimeriVax-JE (see previous discussion).

In these studies, it was noted that the highest immune response to monovalent or tetravalent immunization was elicited by the ChimeriVax-DEN2 virus and this virus also replicated more rapidly than the other three chimeras *in vitro*. To achieve a more balanced antibody response, the dose of DEN2 chimera was lowered to 3 $\log_{10}$ pfu in the tetravalent mixture to reduce its dominant immunogenicity (Guirakhoo *et al.*, 2002). Thirty-six monkeys in nine groups were immunized with 4–5 $\log_{10}$ pfu of monovalent virus, tetravalent YF/DEN vaccine (containing 4.5, 3.0, 3.6, and 4.4 $\log_{10}$ pfu of each virus, YF/DEN1 to-DEN4 viruses, respectively), tetravalent wild type (WT) DEN formulation or unpassaged YF 17D vaccine. Thirty-two of 36 animals became viremic. The magnitude of viremia in the animals receiving tetravalent YF/DEN was similar to that of YF 17D, but significantly lower than that induced by wild-type DEN viruses. After a single dose, monkeys developed high levels of neutralizing antibodies against homologous chimeric viruses as well as to heterologous wild-type DEN viruses isolated in different geographical regions. Administration of a second dose of tetravalent vaccine 2 months later increased titers to both homologous and heterologous viruses. The lower dose of YF/DEN-2 virus in the tetravalent formulation resulted in a more balanced response against dengue 1, 2, and 3 viruses, but somewhat higher against chimeric dengue 4 virus (Table IV), suggesting that further formulations for dose adjustments need to be tested in monkeys to identify an optimal formulation for humans.

A phase 1 trial of ChimeriVax-DEN2 vaccine has recently been completed. This was a randomized, double-blind, controlled, single-center outpatient study in 42 YF-naïve healthy adult, male and female subjects. Groups of 14 subjects received 5.0 or 3.0 $\log_{10}$ pfu of ChimeriVax-DEN2 vaccine, and 14 subjects received YF-VAX as a control for safety and viremia. The study also comprised an open component in which antibody response to ChimeriVax-DEN2 vaccination (5.0 $\log_{10}$ pfu) was evaluated in 14 YF-immune subjects.

There were no serious adverse events. All except 1 subject in the ChimeriVax-DEN2 5.0 $\log_{10}$ pfu YF naïve group reported one or more non-serious adverse events. The profile of adverse events following vaccination with ChimeriVax-DEN2 was similar to that with YF-VAX. Overall, the most frequent individual adverse events were headache, myalgia and fatigue. There was evidence to suggest a dose-response

relationship for ChimeriVax-DEN2 in YF-naïve subjects with respect to the incidence of headache adverse events.

Both YF-VAX and ChimeriVax-DEN2 induced transient, low-level viremias. In naïve subjects, viremia following YF-VAX did not exceed 2.2 $\log_{10}$ pfu/mL and that to ChimeriVax-DEN2 did not exceed 1.8 $\log_{10}$ pfu/mL. The mean peak viremia and area under the curve of viremia following YF-VAX were higher than those observed in the ChimeriVax-DEN groups, but viremia tended to be of longer duration in the latter.

One hundred percent of subjects receiving ChimeriVax and 92% of subjects receiving YF-VAX seroconverted on neutralization test. There was no evidence of a dose-response relationship with respect to the DEN2 seroconversion rate in subjects vaccinated with ChimeriVax-DEN2. Previous YF immunization had no effect on the DEN2 seroconversion response.

There was no significant difference in geometric mean neutralizing antibody titers at day 31 in YF-naïve subjects vaccinated with 5.0 $\log_{10}$ pfu or 3.0 $\log_{10}$ pfu ChimeriVax-DEN2 (geometric mean titers 1:637 vs. 1:971) or between YF-naïve and YF-immune subjects vaccinated with ChimeriVax-DEN2 5.0 $\log_{10}$ pfu (1:971 vs. 1:601) ($P = 0.99$, ANOVA).

The results of this trial provided the proof of principle for the yellow fever/dengue chimeric vaccine approach in humans. A trial of tetravalent vaccine prepared by formulating a mixture of ChimeriVax vaccine candidates against all four serotypes is planned.

C. Chimeric YF/West Nile Vaccine

A similar vaccine against West Nile (WN) virus has been constructed, using the prM-E genes of wild-type (NY99) WN virus inserted into the YF 17D infectious clone. The construction was achieved using the standard two-plasmid system (Arroyo *et al.*, 2001b; Monath, 2001). The YF/WN chimera is significantly less neurovirulent for mice than YF 17D vaccine. A single inoculation of mice or hamsters elicited strong neutralizing antibody titers and protected against IP and IC challenge with WN virus. The feasibility of immunizing horses has also been demonstrated (Bowen R., Arroyo J., Monath T. P., unpublished result). Horses given two doses of the chimeric vaccine developed neutralizing antibody and were protected against lethal challenge with WN virus introduced directly into the central nervous system. Clinical development of the new vaccine is under way at Acambis Inc.

VII. Summary

Many arthropod-borne flaviviruses are important human pathogens responsible for diverse illnesses, including YF, JE, TBE, and dengue. Live, attenuated vaccines have afforded the most effective and economical means of prevention and control, as illustrated by YF 17D and JE SA14-14-2 vaccines. Recent advances in recombinant DNA technology have made it possible to explore a novel approach for developing live attenuated flavivirus vaccines against other flaviviruses. Full-length cDNA clones allow construction of infectious virus bearing attenuating mutations or deletions incorporated in the viral genome. It is also possible to create chimeric flaviviruses in which the structural protein genes for the target antigens of a flavivirus are replaced by the corresponding genes of another flavivirus. By combining these molecular techniques, the DNA sequences of DEN4 strain 814669, DEN2 PDK-53 candidate vaccine and YF 17D vaccine have been used as the genetic backbone to construct chimeric flaviviruses with the required attenuation phenotype and expression of the target antigens. Encouraging results from preclinical and clinical studies have shown that several chimeric flavivirus vaccines have the safety profile and satisfactory immunogenicity and protective efficacy to warrant further evaluation in humans. The chimeric flavivirus strategy has led to the rapid development of novel live-attenuated vaccines against dengue, TBE, JE, and West Nile viruses.

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ADVANCES IN VIRUS RESEARCH, VOL 61

PROSPECTS FOR ANTIVIRAL THERAPY

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I. Introduction

Currently, there is no specific antiviral therapy available for the treatment of infections with flaviviruses. Symptomatic treatment and intensive medical care are essential to improve the chance of survival in severely infected patients. Early diagnosis of the infection (i.e., before the onset of serious disease) together with an early start of symptomatic treatment, favor survival. Severe flavivirus infections are primarily characterized by encephalitic or hemorrhagic symptoms. The World Health Organization (WHO) has prepared a technical guide in which the treatment of patients displaying either of the symptoms has been addressed (World Health Organization, 1979, 1982).

II. Targets for Antiviral Therapy

A. Viral Targets

1. Introduction

As discussed in other chapters, the replication of flaviviruses depends on the proper function of viral enzymes and cofactors, which are encoded by the non-structural genes. Consequently, these proteins

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are the primary and most specific targets for the development of selective antiviral agents for the treatment of flavivirus infections.

2. Protease

The flavivirus genome contains a single open reading frame that encodes a large polyprotein of >3000 amino acids. This polyprotein undergoes co- and post-translational proteolytic processing by cellular and viral proteases to yield the individual structural and non-structural proteins (reviewed by Chambers *et al.*, 1990a; Lindenbach and Rice, 2001; Westaway, 1987). These non-structural proteins are viral enzymes and cofactors that are essential for viral replication. The viral protease is therefore an important target for the development of specific and selective anti-flavivirus compounds (reviewed by Leyssen *et al.*, 2000).

The crystal structure of the dengue virus serine protease has been reported at 2.1 Å resolution (Krishna Murthy *et al.*, 1999) (Fig. 1). The protease of flaviviruses is constituted by the 167 to 181 N-terminal amino acid residues of the NS3 protein (Li *et al.*, 1999). The NS3

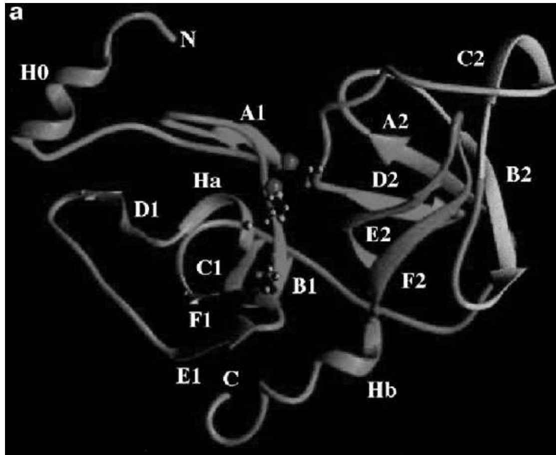

FIG 1. Crystal structure of dengue virus NS3 protease. A Cα trace of the polypeptide chain (*red* and *yellow*) is shown with individual strands and helices labeled. The orientation has been chosen to show the catalytic triad and two water molecules, but the COOH-terminal β-barrel can also be seen. Residues of the catalytic triad are shown as *balls* and *sticks*. Carbon atoms are colored *gray*, nitrogen *cyan*, and oxygen *magenta*. Sections of the chain, which differ from the homologous HCV enzyme, are colored *yellow*. Two water molecules that hydrogen bond to His-51 and Ser-135 and might have significance in catalysis are shown as oversized *purple* spheres (Krishna Murthy *et al.*, 1999). (See Color Insert.)

protease is a serine protease (Bazan and Fletterick, 1989; Gorbalenya *et al.*, 1989a) and belongs to the trypsin superfamily (Gorbalenya *et al.*, 1989a). Four structural conserved regions (Box 1–4 in Table IA) can be identified, in which the serine protease catalytic triad (Bazan and Fletterick, 1989; Chambers *et al.*, 1990b; Gorbalenya *et al.*, 1989a; Valle and Falgout, 1998a) and substrate binding residues (Bazan and Fletterick, 1989; Gorbalenya *et al.*, 1989a; Valle and Falgout, 1998) are located (Valle and Falgout, 1998; Wengler and Wengler, 1993). The NS3 protease requires the presence of the NS2B cofactor for proper functioning (Arias *et al.*, 1993; Chambers *et al.*, 1993; Falgout *et al.*, 1991). Comparison of flavivirus protease sequences with those of virus isolates belonging to the genus pestivirus (e.g., bovine viral diarrhea virus [BVDV]) and hepacivirus (e.g., hepatitis C virus [HCV]) revealed several important sequence similarities (reviewed by Kwong *et al.*, 1999; Ryan *et al.*, 1998). Some differences in the mode of catalysis between flavivirus and hepacivirus proteases have been proposed, however (Murthy *et al.*, 1999). So far, specific inhibitors of the flavivirus NS3 protease have been the subject of limited investigation (Leung, 2001). However, several molecules were shown to inhibit the HCV NS3 protease, including non-peptidic (Chu *et al.*, 1999; Di Marco *et al.*, 2000; Kakiuchi *et al.*, 1998; Sing *et al.*, 2001; Sudo *et al.*, 1997) and peptidomimetic inhibitors (Ingallinella *et al.*, 1998; Johansson *et al.*, 2001; LaPlante *et al.*, 1999; Steinkuhler *et al.*, 1998). As for HIV, peptidomimetic inhibitors (Deeks *et al.*, 1997; Flexner, 1998; Han *et al.*, 2000; Priestley and Decicco, 2000) could be designed by modifying the natural peptide substrates (Drouet *et al.*, 1999; Ingallinella *et al.*, 1998, 2000; Johansson *et al.*, 2001) or by modifying the active loop of mutant eglin c, a well-known protease inhibitor (Martin *et al.*, 1998). The pharmaceutical industry has set up high-throughput *in vitro* screening assays to identify inhibitors of the HCV protease (reviewed by Leyssen *et al.*, 2000). Because of the similarities between the hepacivirus, pestivirus and flavivirus proteases, one may hope that among the compounds that are inhibitors of the HCV or BVDV proteases, some may show inhibitory activity against the flavivirus NS3 protease and thus could serve as a lead in the design of more specific inhibitors of the flavivirus serine protease.

3. Helicase/NTPase

Helicases are enzymes, that unwind double-stranded DNA-DNA, RNA-DNA, or RNA-RNA polynucleotides in an adenosine triphosphate (ATP)–dependent reaction. The function of the helicase of flaviviruses is assumed to be the unwinding of the plus RNA and minus RNA

strands of the genome during the viral replication cycle. The flavivirus helicase is located in the C-terminal three fourths of the NS3 protein (Li *et al.*, 1999; Warrener *et al.*, 1993). It belongs to the family of viral RNA proteins that are part of the helicase superfamily II, also called DEAD/H superfamily (Gorbalenya and Koonin, 1989b; Gorbalenya *et al.*, 1989c; reviewed by Kadare and Haenni, 1997; Koonin, 1992; Lain *et al.*, 1989). The flavivirus NS3 helicase contains seven conserved sequences, denominated I, Ia, II, III, IV, V and VI, of which box I ("A"-site) and box II ("B"-site) constitute the NTP-binding motif (Gorbalenya *et al.*, 1989c; Gorbalenya and Koonin, 1989b; reviewed by Kadare and Haenni 1997) (Table IB). The NTPase activity of the West Nile virus (WNV) (Wengler, 1991) and yellow fever virus (YFV) (Warrener *et al.*, 1993) helicase has been shown to increase significantly in the presence of single-stranded RNA. The crystal structure of the helicase domain of the HCV NS3 (either with or without a bound single-stranded oligonucleotide) has been reported (Kim and Caron, 1998a; Kim *et al.*, 1998b; Yao *et al.*, 1997) (Fig. 2). There may be several potential sites for the interaction of small-molecule inhibitors, including the binding site for ATP and for the single- and double-stranded polynucleotide. These sites are believed to exist in both "open" and "closed" conformations (Fig. 3). Trapping the enzyme in either of these conformations may block the activity of the enzyme (Kim and Caron, 1998a).

4. RNA Triphosphatase, Methyltransferase and Capping

The 5′-terminus of the flavivirus RNA genome contains a type 1 cap structure (m^7GpppA_mpG) (Cleaves and Dubin, 1979; Wengler and Gross, 1978). Because flaviviruses replicate in the cytoplasm and the cellular capping machinery is located in the nucleus, capping of flavivirus RNA may possibly be carried out by viral enzymes. Formation of this cap would require the consecutive action of three enzymes: (1) an RNA triphosphatase for partial dephosphorylation of the 5′-terminal guanosine triphosphate (GTP), (2) a guanylyltransferase for addition of the cap, and (3) a methyltransferase for methylation at the 2′O position of the 5′ penultimate adenosine and the N7 methylation of the ultimate guanosine (Wengler and Wengler, 1993). The exact mechanism of flavivirus genomic RNA capping has yet to be unraveled. The presence of both an RNA triphosphatase domain in the NS3 protein (Wengler and Wengler, 1993) and a methyltransferase domain in the NS5 protein (Koonin, 1993) has been confirmed. So far, no viral guanylyltransferase has been found to be encoded by the

TABLE IA
Conserved Motifs in the NS3 Gene (Serine Protease) of Flaviviruses

Virus	BOX 1	BOX 2	BOX 3	BOX 4
MODV	EGVFHTMWHVTRGSALRVRG	EEDLVAYNGTW	PLDFPPGSSGSPVINAN	GEVVGLYGNGVLHGDT-YCS
APOIV	QGVFHTMWHVTRGSVLSING	RDDLISYNGPW	PIDHPPGTSGSPIITKD	GHVVGLYGNGVVHGET-YCS
MMLV	EGSFHTMWHVTRGATLRIGD	TEDLISYNGGW	PLDFPPGTSGSPIITSS	GHVVGLYGNGIIHGDV-YCS
RBV	GNSFHTMWHVTRGAVLNICG	TEDLISYNGGW	PLDFPPGSSGSPIISSD	GKIIGLYGNGVLHGDT-YCS
POWV	KGVLHTMWHVTRGAALSVEG	REDVVCYGGAW	PIDLPRGTSGSPIINAQ	GDVLGLYGNGLKSNDV-YIS
LGTV	KGVLHTMWHVTRGAALLVDG	REDVVCYGGAW	PIDLAKGTSGSPIMNSQ	GEVVGLYGNGLKTNDT-YVS
TBEV-V	KGVLHTMWHVTRGAALSIND	KEDVVCYGGAW	PIDLAKGTSGSPILNAQ	GAVVGLYGNGLKTNET-YVS
TBEV-S	KGVLHTMWHVTRGAALSIDD	KEDVVCYGGAW	PIDLAKGTSGSPILNSQ	GVVVGLYGNGLKTNET-YVS
TBEV-N	KGVLHTMWHVTRGAALSIDD	REDVVCYGGAW	PIDLVKGTSGSPILNAQ	GVVVGLYGNGLKTNET-YVS
TBEV-H	KGVLHTMWHVTRGAALSIDD	REDVVCYGGAW	PIDLVKGTSGSPILNAQ	GVVVGLYGNGLKTNET-YVS
LIV	KGVLHTMWHVTRGAALSIDD	KEDVVCYGGAW	PIDLAKGTSGSPILNAQ	GVVVGLYGNGPKTNES-YVS
YFV	GGVFHTMWHVTRGAFLVRNG	KEDLVAYGGSW	ALDYPSGTSGSPIVNRN	GEVIGLYGNGILVGDNSFVS
DENV-1	EGVFHTMWHVTRGAVLMYQG	KKDLISYGGGW	ALDFKPGTSGSPIVNRE	GKIVGLYGNGVVTTSGTYVS
DENV-2	EGTFHTMWHVTRGAVLMHKG	KKDLISYGGGW	SLDFSPGTSGSPIIDKK	GKVVGLYGNGVVTRSGAYVS
DENV-3	EGVFHTMWHVTRGAVLTHNG	KKDLISYGGGW	ALDFKPGTSGSPIINRE	GKVVGLYGNGVVTKNGGYVS
DENV-4	EGVFHTMWHVTRGSVICHET	RNDMISYGGGW	TLDFKPGTSGSPIINRK	GKVIGLYGNGVVTKSGDYVS
SLEV	EGVFHTMWHATEGAVLRNGE	RNDLISYGGPW	TLDFPKGTSGSPIINKK	GEIIGLYGNGVLIGQG-YVS
WNV	EGVFHTLWHTTKGAALMSGE	KEDRLCYGGPW	TLDYPTGTSGSPIVDKN	GDVIGLYGNGVIMPNGSYIS
KUNV	EGVFHTLWHTTKGAALMSGE	KEDRLCYGGPW	TLDFPTGTSGSPIVDKN	GDVIGLYGNGVIMPNGSYIS
JEV	ENVFHTLWHTTRGAAIMSGE	KEDRIAYGGPW	SLDYPRGTSGSPILDSN	GDIIGLYGNGVELGDGSYVS
MVEV	EGVFHTLWHTTRGAAIMSGE	KEDRVTYGGPW	SLDYPIGTSGSPIVNSN	GEIIGLYGNGVILGNGAYVS
CFAV	NGVFHTLMHVTRGEPVKWRG	LRDVVSYGGPW	GDDFGKGSSGSPFFIN-	GEPVGFYGFGFYVNGI-YRS
Cat res	H	D	S	
Subst res			D*	Y NG S
Hcons res	H G	D	G SG P	G G S
Ucons res	H	D	G SG	

(Bazan and Fletterick, 1989); (Li *et al.*, 1999); * = not highly conserved.

TABLE IB
Conserved Motifs in the NS3 Gene (Helicase/NTPase) of Flaviviruses

Virus	Motif I	Motif IA	Motif II	Motif III	Motif IV	Motif V	Motif VI
MODV	IVDAHPGSGKTHRILP	RTLVLAPTRIVVKEM	VIIMDEAH	VLMTATPP	AWFVPS	FILTTDISEMGANLDVERVI	TPASAAQRRGRVGR
APOIV	VIDAHPGSGKTKKILP	RTLVLAPTRVVVKEM	LVIMDEAH	VLMTATPP	VWFVPS	FILTTDISEMGANFDVDRVI	TPASAAQRRGRVGR
MMLV	VIDAHPGSGKTHKILP	RTLVLAPTRVVIKEM	LIIMDEAH	VLMTATPP	AWFVPS	FILTTDISEMGANFDVERVI	TPASAAQRRGRVGR
RBV	VIDAHPGSGKTHRILP	RTLVLAPTRVVIKEM	VIIMDEAH	VLMTATPP	AWFVPS	FILTTDISEMGANLDVDRVI	TPASAAQRRGRVGR
POWV	VLDMHPGSGKTHRVLP	RTVVLAPTRVVLKEM	VAIMDEAH	VLMTATPP	AWFVPS	FVVTTDISEMGANLDVNRVI	TTASAAQRRGRVGR
LGTV	VLDMHPGSGKTHRVLP	RTLVLAPTRVVLREM	VAIMDEAH	VLMTATPP	AWFVPS	FVVTTDISEMGANLDVTRVI	TTASAAQRRGRVGR
TBEV-V	VLDMHPGSGKTHRVLP	RTLVLAPTRVVLKEM	VAIMDEAH	VLMTATPP	AWFVPS	FVVTTDISEMGANLDVSRVI	TTASAAQRRGRVGR
TBEV-S	VLDMHPGSGKTHRVLP	RTLVLAPTRVVLKEM	VAIMDEAH	VLMTATPP	AWFVPS	FVVTTDISEMGANLDVSRVI	TTASAAQRRGRVGR
TBEV-N	VLDMHPGSGKTHRVLP	RTLVLAPTRVVLKEM	VAIMDEAH	VLMTATPP	AWFVPS	FVVTTDISEMGANLDVSRVI	TTASAAQRRGRVGR
TBEV-H	VLDMHPGSGKTHRVLP	RTLVLAPTRVVLKEM	VAIMDEAH	VLMTATPP	AWFVPS	FVVTTDISEMGANLDVSRVI	TTASAAQRRGRVGR
LIV	VLDMHPGSGKTHRVLP	STLVLAPTRVVLKEM	VAIMDEAH	VLMTATPP	AWFVPS	FVVTTDISEMGANLDVSRVI	TTASAAQRRGRVGR
YFV	VLDFHPGAGKTRRFLP	RTLVLAPTRVVLSEM	VIIMDEAH	ILMTATPP	AWFLPS	FILATDIAEMGANLCVERVL	SASSAAQRRGRIGR
DENV-1	IMDLHPGSGKTRRYLP	RTLVLAPTRVVASEM	MIIMDEAH	IFMTATPP	VWFVPS	YVVTTDISEMGANRADRVIF	TVASAAQRRGRIGR
DENV-2	IMDLHPGAGKTKRYLP	RTLILAPTRVVAAEM	LIIMDEAH	IFMTATPP	VWFVPS	FVVTTDISEMGANFKAERVI	THSSAAGRRGRIGR
DENV-3	IMDLHPGSGKTRKYLP	RTLILAPTRVVAAEM	LIIMDEAH	IFMTATPP	VWFVPS	FVVTTDISEMGANFIADRVI	TVASAAQRRGRVGR
DENV-4	IMDLHPGAGKTKRILP	RTLILAPTRVVAAEM	LIVMDEAH	IFMTATPP	VWFVPS	FVVTTDISEMGANFRAGRVI	TPASAAQRRGRIGR
SLEV	VLELHPGAGKTRKVLP	RTAVLAPTRVVACEI	VYIMDEAH	IFMTATPP	VWFVPS	FVVTTDISEMGANFGAHRVI	TSASAAQRRGRIGR
WNV	VLDLHPGAGKTRKILP	RTAVLAPTRVVAAEM	LFIMDEAH	IFMTATPP	VWFVPS	FVYTTDISEMGANFKASRVI	TAASAAQRRGRIGR
KUNV	VLDLHPGAGKTRRILP	RTAVLAPTRVVAAEM	LFVMDEAH	IFMTATPP	VWFVPS	FVVTTDISEMGANFKASRVI	TAASAAQRRGRTGR
JEV	VLDLHPGSGKTRKILP	RTAVLAPTRVVAAEM	LFVMDEAH	IFMTATPP	VWFVAS	FVITTDISEMGANFGASRVI	TSASAAQRRGRVGR
MVEV	VLDLHPGAGKTRRILP	RTAVLAPTRVVAAEM	LFVMDEAH	IFMTATPP	VWFVAS	FVITTDISEMGANFGASRVI	TSASAAQRRGRVGR
CFAV	FVTWHPGKGKTRKVIV	RTVILTPTRVVMAEV	MIIMDECH	IYLSATPP	ILFVPS	LVISTDISEMGANLGVDLVI	TTSSMIQRRGRTGR
Ucons res	**G GKT**		**D**				
Consensus	**bbxxxxGSGKTxxxbP**	**RxbuuxPTRxuxxEb**	**bbbbDExH**	**bxuTATPP**	**ubbuPS**	**bubxTDbxExGuxbxxxxuu**	**TxxxxxQRxGRuGR**

(Kadare and Haenni, 1997); b = bulky hydrophobic residue (aliphatic or aromatic), u = bulky aliphatic residue, x = any residue.

TABLE IC

CONSERVED MOTIFS IN THE NS3 GENE (PUTATIVE RNA TRIPHOSPHATASE DOMAIN) OF FLAVIVIRUSES

Virus		5'TRE
MODV	KEHLVTD	ERLQPVW
APOIV	KEHEIAD	NILRPVW
MMLV	KENLVTD	HKLAPVW
RBV	NQHLVSD	RQLQPVW
POWV	DENAIDG	RKLKPIW
LGTV	EENAVDE	RTLRPVW
TBEV-V	EENAVDE	RTLRPVW
TBEV-S	EENTVDE	RTLRPVW
TBEV-N	EANAVDE	RTLRPVW
TBEV-H	EANAVDE	RTLRPVW
LIV	EANAVDE	RTLRPVW
YFV	EEHEILN	KPLRPRW
DENV-1	RNNQVLE	KKLRPRW
DENV-2	RNNQILE	KKLKPRW
DENV-3	KNNQILE	KKLRPRW
DENV-4	RNNQILE	KKLRPRW
SLEV	TNNTILE	KILRPKW
WNV	RTNTILE	KILRPRW
KUNV	RTNTILE	KILRPRW
JEV	RTNAILE	KILKPRW
MVEV	RSNIILE	KVLKPRW
CFAV	GTGKIIE	RFESIEW
Cat res	*	

[Reviewed by Bisaillon and Lemay (1997); Wengler and Wengler, 1993)]

TABLE ID

CONSERVED MOTIFS IN THE NS5 GENE (METHYLTRANSFERASE DOMAIN) OF FLAVIVIRUSES

Virus	MOTIF I	MOTIF II
MODV	VVDLGCGRGGW	ADTVLCDIGES
APOIV	VVDLGSGRGGW	VDTLLCDIGES
MMLV	VVDLGCGRGGW	CDTILCDIGES
RBV	VVDLGCGRGGW	CDTVLCDIGES
POWV	VVDLGCGRGGW	ADTILCDIGES
LGTV	VVDLGCGRGGW	ADAILCDIGES
TBEV-V	VVDLGCGRGGW	ADTIMCDIGES
TBEV-S	VVDLGCGRGGW	ADTIMCDIGES
TBEV-N	VVDLGCGRGGW	ADTVMCDIGES
TBEV-H	VVDLGCGRGGW	ADTVMCDIGES
LIV	VVDLGCGRGGW	ADTIMCDIGES
YFV	VIDLGCGRGGW	CDTLLCDIGES
DENV-1	VIDLGCGRGGW	CDTLLCDIGES
DENV-2	VVDLGCGRGGW	CDTLLCDIGES
DENV-3	VIDLGCGRGGW	CDTLLCDIGES
DENV-4	VVDLGCGRGGW	VDTLLCDIGES
SLEV	VVDLGCGRGGW	ADTVLCDIGES
WNV	VVDLGCGRGGW	SDTLLCDIGES
KUNV	VIDLGCGRGGW	CDTLLCDIGES
JEV	VIDLGCGRGGW	SDTLFCDIGES
MVEV	VVDLGCGRGGW	SDTLLCDIGES
CFAV	VVDLGCGRGGW	CDTIMCDIGES
Ucons res	D G	D
Consensus	bbDbooGxo	

[Koonin, 1993; reviewed by Bisaillon and Lemay (1997)]; u = bulky aliphatic residue, o = small residue, x = any residue

TABLE IE

CONSERVED MOTIFS IN THE NS5 GENE (NUCLEAR LOCALISATION SITES) OF FLAVIVIRUSES

Virus	NLS-1 CK2 NLS-2
MODV	QVKKVMRKVFKWLIERIKTKGGKVRTCTKEEFIQKVRSHA
APOIV	EVKKVMRLVFRWLLNHIKSKGAVVRRCTKEEFINKVNSNA
MMLV	EVKKVMRVVFNWLVHVILKNGGKVRKCTREEFIKKVESHA
RBV	EVKIVMRKVFTWLVKRILKKGGKIRKCTKEEFINKVESHA
POWV	GTRVIMRAVSDWLLEHLSRRA-KVRMCTKDEFIAKVRSNA
LGTV	GTKIIMRAVNDWLLERLVKKS-RPRMCSREEFIAKVRSNA
TBEV-V	GTKVIMRAVNDWILERLAQKS-KPRMCSKEEFIAKVKSNA
TBEV-S	GTKVIMRAVNDWILERLARKS-KPRMCSREEFIAKVKSNA
TBEV-N	GTRVIMRAVNDWILERLAQKS-KPRMCSREEFIAKVKSNA
TBEV-H	GTRVIMRAVNDWILERLAQKS-KPRMCSREEFIAKVKSNA
LIV	GTRVITRAVNDWILERLAQKS-KPRMCSREEFIAKVRSNA
YFV	GTRKIMKVVNRWLFRHLAREK-NPRLCTKEEFIAKVRSHA
DENV-1	GTAQIMEVTARWLWGFLSRNK-KPRICTREEFTRKVRSNA
DENV-2	GTKKLMKITAEWLWKELGKKK-TPRMCTREEFTRKVRSNA
DENV-3	GTRKVMEITAEWLWRTLGRNK-RPRLCTREEFTKKVRTNA
DENV-4	GTRMVMTTTANWLWALLGKKK-NPRLCTREEFISKVRSNA
SLEV	GVAQIMDVTTDWLWDFVAREK-KPRVCTPEEFKAKVNSHA
WNV	GVKYVTNETTNWLWAFLARDK-KPRMCSREEFIGKVNSNA
KUNV	GVKYVLNETTNWLWAFLAREK-RPRMCSREEFIRKVNSNA
JEV	GAKEVLNETTNWLWAHLSREK-RPRLCTKEEFIKKVNSNA
MVEV	GVKTVMDETTNWLWAYTARNK-KARLCTREEFVKKVNSHA
CFAV	HIRRVNRTITKHFIRLFKNRNLRPRILSKEEFVANVRNDA
CK2 site	**S/T**

(Forwood *et al.*, 1999)

TABLE IF

CONSERVED MOTIFS IN THE NS5 GENE (RNA-DEPENDENT RNA POLYMERASE DOMAIN) OF FLAVIVIRUSES

Virus	MOTIF A	MOTIF B	MOTIF C	MOTIF D
MODV	YADDTAGWDTR	GQVVTYALNTLTN	SGDDCVV	LNAMEKVRKD
APOIV	YADDTAGWDTR	GQVVTYALNTLTN	SGDDCVV	LNTMEKTRKD
MMLV	YADDTAGWDTR	GQVVTYALNTLTN	SGDDCVV	LNEMQKIRKD
RBV	YADDTAGWDTR	GQVVTYALNTLTN	SGDDCVV	LNTMEKIRKD
POWV	YADDTAGWDTR	GQVVTYALNTITN	SGDDCVV	LNDMAKVRKD
LGTV	YADDTAGWDTR	GQVVTYALNTITN	SGDDCVV	LNDMAKTRKD
TBEV-V	YADDTAGWDTK	GQVVTYALNTLTN	SGDDCVV	LNDMAKTRKD
TBEV-S	YADDTAGWDTK	GQVVTYALNTLTN	SGDDCVV	LNDMAKTRKD
TBEV-N	YADDTAGWDTK	GQVVTYALNTLTN	SGDDCVV	LNDMAKTRKD
TBEV-H	YADDTAGWDTK	GQVVTYALNTLTN	SGDDCVV	LNDMAKTRKD
LIV	YADDTAGWDTK	GQVVTYALNTLTN	SGDDCVV	LNDMAKTRKD
YFV	YADDTAGWDTR	GQVVTYALNTITN	SGDDCVV	LNAMSKVRKD
DENV-1	YADDTAGWDTR	GQVGTYGLNTFTN	SGDDCVV	LNDMGKVRKD
DENV-2	YADDTAGWDTR	GQVGTYGLNTFTN	SGDDCVV	LNDMGKVRKD
DENV-3	YADDTAGWDTR	GQVGTYGLNTFTN	SGDDCVV	LNDMGKVRKD
DENV-4	YADDTAGWDTR	GQVGTYGLNTFTN	SGDDCVV	LNDMGKVRKD
SLEV	YADDTAGWDTR	GQVVTYALNTFTN	SGDDCVV	LNNMSKIRKD
WNV	YADDTAGWDTR	GQVVTYALNTFTN	SGDDCVV	LNAMSKVRKD
KUNV	YADDTAGWDTR	GQVVTYALNTFTN	SGDDCVV	LNAMSKVRKD
JEV	YADDTAGWDTR	GQVVTYALNTFTN	SGDDCVV	LNAMSKVRKD
MVEV	YADDTAGWDTR	GQVVTYALNTFTN	SGDDCVV	LNAMSKVRKD
CFAV	IADDIAGWDTK	GQVVTYALNTITN	AGDDCVV	LELTGKTRKN
Ucons res	**D**	**G**	**DD**	**K**
Consensus	**DxxxxxD**	**GxxxTxxxNT**	**GDD**	

[(Bruenn, 1991); reviewed by O'Reilly and Kao (1998); (Poch *et al.*,1989)]

flavivirus genome, which suggests that this function may be ensured by a cellular enzyme.

Flavivirus RNA triphosphatase activity has been allocated to a 50 kDa protein generated by proteolytic treatment of the WNV NS3, and has been tentatively assigned to a third functional domain located at the ultimate C-terminal end of the NS3 protein (Wengler and Wengler, 1993). This domain is characterized by the presence of a highly conserved 5′-terminal recognition element (5′-TRE), which is assumed to be involved in positioning of the substrate RNA 5′-terminal triphosphate into the active center of the enzyme (Wengler and Wengler, 1993) (Table IC). The sequence of the 5′-TRE was found to be highly similar to the vaccinia virus D1 subunit LKPR sequence, which is known to be involved in the capping of vaccinia virus RNA transcripts and which possesses RNA triphosphatase activity (reviewed by Bisaillon and Lemay, 1997). However, there have been no further reports that support these results and, extend these findings to other flaviviruses. Except for the 5′-TRE, also a somewhat degenerated motif has been identified in the flavivirus NS3, vaccinia D1 subunit and reovirus λ1 protein (reviewed by Bisaillon and Lemay, 1997) (Table IC). This motif has been shown to be essential for the RNA triphosphatase activity of the vaccinia D1 subunit (Yu and Shuman, 1996). More research is required to prove that these motifs are essential for RNA

Footnote to Tables IA–1F: GenBank accession numbers and abbreviations of the viruses: *Louping ill virus* (LIV; Y07863), *Tick-borne encephalitis virus* (TBEV-H, Hypr strain, U39292; TBEV-N, Neudoerfl strain, U27495; TBEV-V, Vasilchenko strain, L40361 and TBEV-S, Sofjin strain, P07720), *Langat virus* (LGTV, AF253419), *Powassan virus* (POWV; L06436), *Apoi virus* (APOIV; AF160193), *Modoc virus* (MODV, AJ242984), *Rio Bravo virus* (RBV; AF144692), *Yellow fever virus* (YFV; X03700), *Dengue fever virus* (serotype 1, DENV-1, U88536; serotype 2, DENV-2, AF038403; serotype 3, DENV-3, M93130 and serotype 4, DENV-4, M14931), *Saint Louis encephalitis virus* (SLEV; M16614, AH009306, AF013416), *Kunjin virus* (KUNV; D00246), *West Nile virus* (WNV; M12294), *Japanese encephalitis virus* (JEV; M18370), *Murray Valley encephalitis virus* (MVEV; NC_000943) and *Cell fusing agent virus* (CFAV, M91671). All motifs described in the Tables are involved in catalytic function, or are of structural importance. Also, the putative motifs of the RNA triphosphatase have been included in the tables. Amino acid residues conserved among all flaviviruses (2 exceptions were allowed) are printed in bold and are highlighted. Catalytic, substrate binding and ultraconserved residues, as well as known consensus sequences, are indicated at the bottom of the alignments in separate boxes. References are included in each Table. Abbreviations: Ultraconserved residues within the respective enzyme family (Ucons res); highly conserved residues within the family of flaviviruses (Hcons res); catalytic residues (Cat res); substrate binding residues (Subst res); consensus sequence (Consensus).

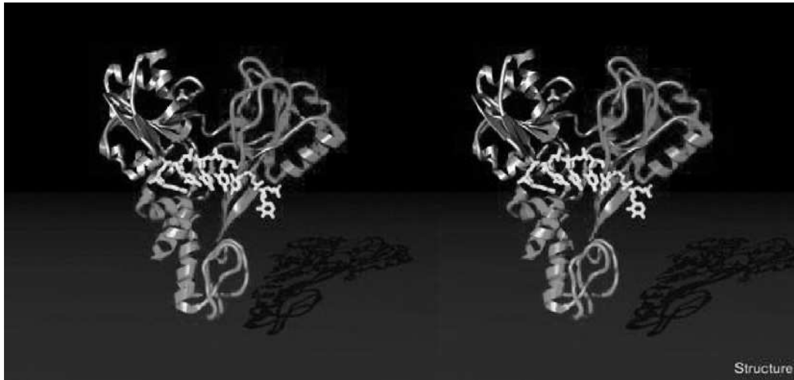

FIG 2. Crystal structure of the HCV NS3 RNA helicase (Kim *et al.*, 1998b). Fold of HCV NS3 helicase domain complexed with ssDNA. Stereo ribbon diagram illustrating the overall fold of the NS3 helicase with bound dU8. Domain 1 is blue, domain 2 red, and domain 3 green. The sulfate ion is shown in light green and the DNA is yellow. (See Color Insert.)

triphosphatase activity in flaviviruses, and that the latter activity is involved in flavivirus RNA capping. A putative flavivirus-encoded methyltransferase has been identified in the N-terminal domain of the NS5 protein. Two highly conserved motifs suggest the requirement of S-adenosylmethionine (SAM) as a methyldonor (Table ID). Motif I represents the characteristic G-loop and Motif II contains an invariant Asp residue (Koonin, 1993; reviewed by Bisaillon and Lemay, 1997). As for the RNA triphosphatase, the structure-function relationship for this viral enzyme has yet to be established.

Because the single-stranded (+) RNA [ss(+)RNA] genome of flaviviruses carries a cap structure, the RNA that is released into the cytosol following uncoating of the viral particle will serve as a template for translation, and thus for the production of structural and non-structural proteins. At a later stage in the replication cycle, capping of new progeny ss(+)RNA genomes will occur. It may therefore be assumed that new progeny viruses that are formed in cells treated with inhibitors of viral capping (such as the SAH hydrolase inhibitors; see subsequent discussion), may have reduced infectivity. However, such inhibitors may have a more profound inhibitory effect on the replication of ss(−)RNA viruses, where initiation of replication cannot proceed until the intermediate ss(+)RNA strand has acquired a complete cap structure.

Thus far, no compounds have been reported that selectively block viral capping by inhibiting the RNA triphosphatase, guanylyltransferase or

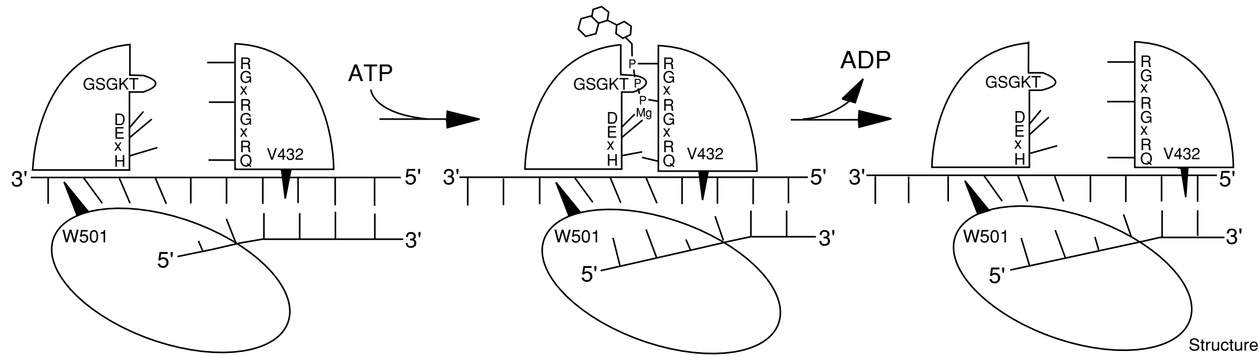

FIG 3. HCV helicase: mechanism schematic (Kim *et al.*, 1998b). The binding of a polynucleotide by NS3 helicase in the absence of ATP leaves a large cleft between domains 1 and 2. Binding of ATP occurs with the β-phosphate binding to residues in motif I (GSGKT) and the γ-phosphate with $Mg^{2}+$ binding to the conserved acidic residues in motif II (DECH). This results in the closing of the interdomain cleft and the binding of conserved arginines in motif VI (QRRGRTGR) to the ATP phosphates. Val432 and Trp501 disrupt base stacking at either end of the single-stranded region. Closure of the interdomain cleft leads to translocation of the single strand in the 5′ to 3′ direction and forces several bases to slip past Trp501. Hydrolysis of ATP facilitates opening of the cleft and release of ADP. The orientation of Trp501 favors movement of the polynucleotide in only one direction such that opening of the gap results in net movement of the helicase in a 3′ to 5′ direction.

methyltransferase. However, the latter can be indirectly targeted by depleting the SAM pools in the host cells through blockade of the cellular S-adenosylhomocysteine (SAH) hydrolase (see subsequent discussion). Ribavirin 5′-triphosphate, which may be viewed as a GTP analogue, has been hypothesized to inhibit the cellular guanylyltransferase (see subsequent discussion) (Goswami *et al.*, 1979). However, this has not been followed up or confirmed.

5. RNA-dependent RNA Polymerase

Another main target for the development of agents that could inhibit flavivirus replication is the RNA-dependent RNA polymerase (RdRp). The RdRp domain is located in the C-terminal part of the NS5 protein and is separated from the N-terminal methyltransferase domain (see previous discussion) by two nuclear localization sites (NLS) (Table IE). The second NLS contains a serine or threonine residue that can be phosphorylated by CK2, a serine/threonine protein kinase (Forwood *et al.*, 1999; reviewed by Lindenbach and Rice, 2001; Reed *et al.*, 1998).

The crystal structure of the RdRp of flaviviruses has not yet been determined. However, the crystal structure of the RdRp of HCV may provide important information (Lesburg *et al.*, 1999) (Fig. 4). The three-dimensional structure of the HCV RNA polymerase is, like most other RNA or DNA polymerases, organized around a central cleft which contains the catalytic domain, divided into subdomains termed “palm,” “fingers,” and “thumb” because the enzyme resembles a right hand (Bressanelli *et al.*, 1999; Lesburg *et al.*, 1999). In the RNA-dependent RNA polymerase domain, eight motifs can be defined of which four are known to be present in all classes of RNA polymerases and reside in the catalytic portion of the “palm” domain (Table IF). Motif A is involved in magnesium coordination and possibly also sugar selection; Motif B has been identified to be the core motif for nucleotide binding and is also involved in sugar selection; Motif C is involved in the coordination of the Mg^{2+} and is the proposed core motif for the catalytic RNA polymerase activity, and Motif D completes the palm core structure of the polymerase (Bruenn, 1991; reviewed by O’Reilly and Kao, 1998; Poch *et al.*, 1989).

Considering the likely importance of the RdRp as an antiviral target for the treatment of HCV infections, the pharmaceutical industry has initiated screening programs to identify inhibitors of the RdRp of HCV. The intense search for selective inhibitors of the HCV RdRp may possibly also help to discover selective inhibitors of flavivirus RdRps.

One of the potential mechanisms that has been suggested by which ribavirin may exert anti-HCV activity is the usage by the HCV RdRp

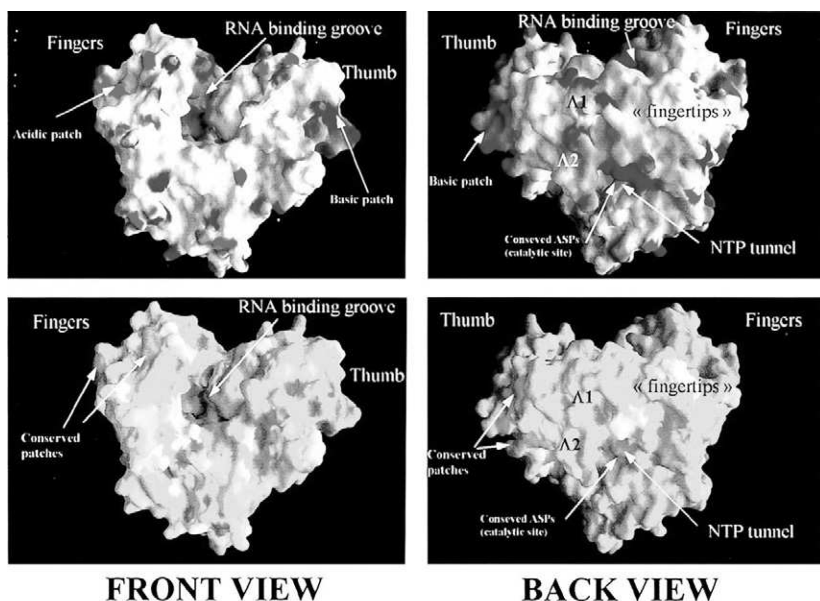

FIG 4. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus (Bressanelli *et al.*, 1999). The molecular surface of the HCV RdRp, front view (*left*) and back view (*right*). Electrostatic potential calculated on the molecular surface. Positive and negative charges are indicated in *blue* and *red*, respectively. The positively charged groove along which the RNA would bind during viral replication is apparent in the front view. The region of the thumb at the very C-terminus of the catalytic domain of NS5B is also positively charged. In the back view, the lower ridge of the "bridge" formed by loops A1 and A2 is seen to have a strong positive charge. This area corresponds to the "tunnel" where NTP would access to the polymerizing complex. The red patch underneath the tunnel corresponds to the strictly conserved aspartic acids that are known to coordinate the catalytic Mg^{2+} ions. (See Color Insert.)

of ribavirin 5′-triphosphate as an alternative substrate. This would cause an increase in mutation frequency and hence the production of unviable virus (Maag *et al.*, 2001). The discovery of VP32947 (3-[((2-dipropylamino)ethyl)thio]-5H-1,2,4-triazino[5,6-b]indole), a compound that selectively inhibits the RdRp of pestiviruses, indicates that it may also be possible to develop selective antivirals targeted at the viral RdRp of HCV and flaviviruses (Baginski *et al.*, 2000).

6. *Conclusions*

Viral targets such as the serine protease, the helicase/NTPase, the RdRp and, perhaps, the capping machinery, offer the possibility to develop selective inhibitors of flavivirus replication. Alignment of the

amino acid sequence of 23 flaviviruses, representing all clades of the genus Flavivirus, indicates that the amino acid residues of the protease, helicase/NTPase, methyltransferase and RNA polymerase that are responsible for catalytic function and substrate binding are highly conserved amongst all flaviviruses (Tables IA–E). Thus, a molecule that selectively inhibits the replication of one flavivirus by targeting one of the viral enzymes involved in viral replication may stand a good chance of being active against other flaviviruses as well.

B. Cellular Targets

1. Introduction

In addition to the virus-specific enzymes, cellular enzymes may also be valuable targets for therapeutic intervention against flaviviruses. This includes enzymes such as (i) the inosine monophosphate (IMP) dehydrogenase, the S-adenosylhomocysteine (SAH) hydrolase, the orotidylate monophosphate (OMP) decarboxylase and the cytidine triphosphate (CTP) synthetase (De Clercq, 1993), which play a key role in the supply of "building blocks," needed by the viral enzymes to produce progeny virus; and (ii) enzymes that are involved in the "maturation" of the virus; for example, glycosylation of the envelope protein. Inhibitors interacting with such targets may undoubtedly have negative effects on healthy, uninfected cells. However, targeting such cellular enzymes for a short time during an acute flavivirus infection may be sufficient to reduce viral replication to such an extent to allow the immune system the time to build up a protective response.

2. Inosine Monophosphate Dehydrogenase

The IMP dehydrogenase plays a key role in the biosynthetic pathway of purine nucleotides by catalyzing the conversion of IMP to xanthosine monophosphate (XMP), which in turn serves as a substrate for the production of guanosine monophosphate (GMP) by GMP synthetase. Because (i) guanosine nucleotides are less readily available in the cell as compared to adenosine nucleotides, (ii) there is no alternative biosynthetic pathway of guanosine nucleotides, and (iii) production of viral genomic RNA requires a comparable amount of both ATP and GTP, inhibiting the IMP dehydrogenase and thus interfering with the supply of GTP provides an alternative, non-viral targeted strategy for inhibiting flavivirus replication.

The IMP dehydrogenase contains both a nucleotide substrate- and a NAD$^+$-binding pocket. Nucleotide analogues, such as ribavirin [1-(β-D-ribofuranosyl)-1,2,4,-triazole-3-carboxamide] (Huggins *et al.*, 1984; Jordan *et al.*, 2000; Kirsi *et al.*, 1983; Koff *et al.*, 1982; Leyssen *et al.*, 2000; Neyts *et al.*, 1996; Smee *et al.*, 1992), the ribavirin analogue EICAR (5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide) (Leyssen *et al.*, 2000; Neyts *et al.*, 1996), tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) (Huggins *et al.*, 1984; Kirsi *et al.*, 1983; Leyssen *et al.*, 2000; Neyts *et al.*, 1996), and selenazofurin (2-β-D-ribofuranosylselenazole-4-carboxamide) (Huggins *et al.*, 1984; Kirsi *et al.*, 1983; Leyssen *et al.*, 2000; Neyts *et al.*, 1996), inhibit the replication of several flaviviruses in cell culture assays. Ribavirin and EICAR inhibit IMP dehydrogenase activity after they have been converted to their 5′-monophosphate metabolite (Balzarini *et al.*, 1993). It is as yet not clear to what extent the inhibitory effects of ribavirin and EICAR on IMP dehydrogenase are responsible for the antiviral effects of these compounds. Mycophenolic acid (MPA), an inhibitor of the IMP dehydrogenase that interferes with the function of the coenzyme NAD$^+$, is a very potent *in vitro* inhibitor of flavivirus replication (Leyssen *et al.*, 2000; Neyts *et al.*, 1996). The structurally unrelated compound VX-497 ((S)-N-3-[3-(3-methoxy-4-oxazol-5-yl-phenyl)-ureido]-benzyl-carbamic acid tetrahydro-furan-3-yl-ester), which, like MPA, interacts with the NAD$^+$-binding pocket of the enzyme (Sintchak and Nimmesgern, 2000), has also been shown to inhibit the replication of flaviviruses (Markland *et al.*, 2000). Neither ribavirin nor MPA or VX-497 have so far proven to be of therapeutic benefit in an animal model of flavivirus infection.

3. S-adenosylhomocysteine Hydrolase

The SAH hydrolase is a pivotal enzyme in the regeneration cycle of S-adenosylmethionine (SAM). The latter serves as a methyldonor in methylation reactions such as those required for CAP formation. Inhibition of the SAH hydrolase leads to accumulation of SAH, which serves as an inhibitor of the SAM-dependent methylation reactions, including those required for maturation of viral RNA. A variety of carbocyclic adenosine analogues are assumed to exert their antiviral action through inhibition of the SAH hydrolase. In fact, a close correlation has been detected between the antiviral effects of various carbocyclic and acyclic adenosine analogues and their inhibitory effects on cell free SAH hydrolase (Cools and De Clercq, 1989). These compounds proved to be weak inhibitors of flavivirus replication in plaque reduction assays (Neyts *et al.*, 1996; Tseng *et al.*, 1989) relative to the potent

activity they exert against the replication of ss(−)RNA viruses such as Ebola (Bray *et al.*, 2000; Huggins *et al.*, 1999). As outlined previously, however, SAH hydrolase inhibitors may possibly reduce the infectivity of newly formed virus particles and have a profound effect on second and further replication cycles.

4. Orotidylate Monophosphate Decarboxylase and Cytidine Triphosphate Synthetase

The conversion of OMP to uridine monophosphate (UMP) is catalyzed by the OMP decarboxylase. Inhibition of this enzyme shuts off the supply of pyrimidine nucleotides needed for the synthesis of viral RNA. Inhibitors of the OMP decarboxylase such as pyrazofurin (3-(β-D-ribofuranosyl)-4-hydroxypyrazole-5-carboxamide) (Westhead and Price, 1974) and 6-azauridine (Rizki and Rizki, 1965) were shown to inhibit flavivirus replication in cell culture (Canonico *et al.*, 1982; Neyts *et al.*, 1996).

The CTP synthetase catalyzes the conversion of uridine triphosphate (UTP) to CTP, the key step in the biosynthesis of cytidine nucleotides. Inhibitors of this enzyme, such as carbodine (cyclopentyl cytosine) (De Clercq *et al.*, 1990; Shannon *et al.*, 1981) and cyclopentenyl cytosine (De Clercq *et al.*, 1991b; Glazer *et al.*, 1985), also inhibit flavivirus replication in cell culture (Neyts *et al.*, 1996).

5. α-Glucosidase and Glycosylation

Assembly of progeny virions occurs in close association with intracellular membrane complexes. While being assembled and secreted by the host cell, the proviral particles mature into infectious virus particles (Chambers *et al.*, 1990; Lindenbach and Rice, 2001). During this maturation process, glycosylation of the envelope proteins takes place. α-Glucosidases are responsible for the stepwise removal of terminal glucose residues from the N-glycan chains attached to nascent glycoproteins. This enables the glycoproteins to interact with the endoplasmic reticulum (ER) chaperones calnexin and calreticulin, which bind exclusively to monoglucosylated glycoproteins. Interaction with calnexin is crucial for the correct folding of some, but not all, glycoproteins (Mehta *et al.*, 1998; Rudd and Dwek, 1997). Compounds that interfere with normal glycosylation, and therefore prevent the viral glycoproteins from folding correctly, may influence maturation and secretion of the virus, and thus reduce the infectivity of newly released viral particles (Branza-Nichita *et al.*, 2001).

The iminosugars *N*-butyldeoxynojirimycin (*N*B-DNJ) and *N*-nonyldeoxynojirimycin (*N*N-DNJ) are inhibitors of the ER α-glucosidase

and were shown to inhibit BVDV replication *in vitro* in a dose-dependent manner (Zitzmann *et al.*, 1999). Long-alkyl-chain derivatives of deoxygalactonojirimycin (DGJ), such as *N*7-oxanonyl-6-deoxy-DGJ, do not inhibit the ER α-glucosidase (as DNJ and derivatives do) (Durantel *et al.*, 2001). Castanospermine (CST), another α-glucosidase inhibitor, was shown to cause misfolding of recombinant HCV E1 and E2 glycoproteins expressed in BHK-21 cells and to reduce their association into native dimers (Choukhi *et al.*, 1998). Also CTS and DNJ were shown to affect the morphogenesis and assembly of dengue virus in mouse neuroblastoma cells (Courageot *et al.*, 2000). DNJ has been shown to inhibit JE virus replication in cell culture and confer protective effects against JE infection in a mouse model (Wu *et al.*, 2002). Tunicamycin was found to inhibit the acquisition of native conformation of the envelope protein of Japanese encephalitis virus (JEV) in porcine kidney cells and down-regulated virus maturation inside the cells (Lad *et al.*, 2000). Inhibition of glycosylation, and therefore assembly, maturation, and secretion of the progeny virions may reduce the virulence of flaviviruses.

6. Conclusions

In addition to viral enzymes, cellular enzymes may also be appropriate targets for the design of antiviral agents that impair the replication of flaviviruses. The major disadvantage of such inhibitors is their potential toxicity. Evidence that inhibitors of cellular enzymes may indeed be protective against viral infection stems from the observations that inhibitors of SAH hydrolase protect mice against a lethal Ebola virus infection. Use of compounds that target cellular enzymes would preferentially be limited to a short duration of time during the acute phase of the infection. The reduction in viral replication brought about by such compounds may be sufficient to allow the immune system the time to mount a protective response. Furthermore, as these enzymes are of cellular rather than viral origin, emergence of drug-resistant viruses is less likely to occur.

III. Antiviral Therapy: Past, Present, and Future

A. Ribavirin and Derivatives

1. Introduction

The triazole nucleoside, ribavirin (Virazole, Virazid), was first synthesized in 1972 (Witkowski *et al.*, 1972) and, after more than three decades, still proves to be useful for the treatment of a number of viral

infections. Ribavirin is a broad-spectrum antiviral agent that inhibits a selection of RNA viruses *in vitro* and *in vivo* (Sidwell *et al.*, 1972). Ribavirin is approved for clinical use for the treatment of infections with the respiratory syncytial virus (Fernandez *et al.*, 1986), Lassa fever virus (Fernandez *et al.*, 1986), and, more recently, in combination with interferon, for the treatment of chronic infections with the hepatitis C virus (Reichard *et al.*, 1998). Recently, ribavirin was also shown to cause protection against Nipah virus–associated encephalitis and mortality (Chong *et al.*, 2001). Although ribavirin is effective in cell culture against flaviviruses, the compound causes little or no protective effect in animal models and in the clinical setting. Precise knowledge of the mode of antiviral action of ribavirin may allow the design of more potent and selective analogues. A summary of the data on the antiviral activity of ribavirin and analogues against several flaviviruses is presented in Table II.

2. Pharmacokinetics

Ribavirin has a favorable pharmacokinetic profile: a distribution volume of 2000 L (Glue, 1999), extensive absorption (Glue, 1999), no binding to plasma proteins (Glue, 1999), and a serum half-life of 29–60 hours following multiple dosing (Glue, 1999). However, ribavirin also suffers from a number of drawbacks such as a relatively low oral bioavailability (approximately 50%) (Paroni *et al.*, 1989; Preston *et al.*, 1999) and poor penetration of the blood-brain barrier (Huggins, 1989). Major side effects are headache, fatigue, and a dose-dependent reversible anemia. The probable cause for the anemia is a decrease in erythrocyte half-life (Canonico *et al.*, 1984) and inhibition of the release of erythrocytes from the bone marrow (Canonico *et al.*, 1984). The dephosphorylation rate of ribavirin 5′-triphosphate (RTP), the active metabolite of ribavirin, in red blood cells is very limited (Page and Connor, 1990), which results in intracellular accumulation of this metabolite (Connor *et al.*, 1993; Lertora *et al.*, 1991). This accumulation eventually results in hemolysis (Canonico *et al.*, 1984). Ribavirin is also suspected to be teratogenic and is contraindicated in pregnant and lactating females because of free passage of the drug into breast milk (Knight and Gilbert, 1988).

3. Mechanism of Action against Flaviviridae

It still remains controversial as to how ribavirin exactly exerts its antiviral activity. Although from its structural formula ribavirin may resemble a pyrimidine nucleoside, radiographic studies showed

TABLE II
In Vitro Activity of Ribavirin and Analogues against Flaviviruses

Virus (strain)	Cell type	IC_{50} (μg/ml)	References
Ribavirin			
BZV (H336)	HeLa	24	(Smee *et al.*, 1987)
DENV type-2	Vero	49	(Leyssen *et al.*, 2000)
JEV (Nakayama)	Vero-76	42	(Kirsi *et al.*, 1983)
JEV (Nakayama)	Vero-76	65	(Huggins *et al.*, 1984)
WNV (NY 1999)	Human oligodendroglia	15	(Jordan *et al.*, 2000)
YFV (17D)	Vero	28	(Leyssen *et al.*, 2000)
YFV (17D)	Vero	28	(Neyts *et al.*, 1996)
YFV (Asibi)	LLC-MK_2	250	(Canonico *et al.*, 1982)
YFV (Asibi)	LLC-MK_2	46	(Kirsi *et al.*, 1983)
YFV (Asibi)	LLC-MK_2 or Vero E6	8	(Huggins *et al.*, 1984)
EICAR			
DENV type-2	Vero	2.4	(Leyssen *et al.*, 2000)
YFV (17D)	Vero	0.8	(Leyssen *et al.*, 2000)
YFV (17D)	Vero	0.8	(Neyts *et al.*, 1996)
Selenazofurin			
DENV type-2	Vero	10	(Leyssen *et al.*, 2000)
JEV (Nakayama)	Vero-76	73	(Kirsi *et al.*, 1983)
JEV (Nakayama)	Vero-76	3	(Huggins *et al.*, 1984)
YFV (17D)	Vero	3.6	(Leyssen *et al.*, 2000)
YFV (17D)	Vero	2.2	(Neyts *et al.*, 1996)
YFV (Asibi)	LLC-MK_2	9.2	(Kirsi *et al.*, 1983)
YFV (Asibi)	LLC-MK_2 or Vero E6	0.08	(Huggins *et al.*, 1984)
Tiazofurin			
JEV (Nakayama)	Vero-76	3	(Kirsi *et al.*, 1983)
JEV (Nakayama)	Vero-76	73	(Huggins *et al.*, 1984)
YFV (Asibi)	LLC-MK_2	0.005	(Kirsi *et al.*, 1983)
YFV (Asibi)	LLC-MK_2 or Vero E6	0.08	(Huggins *et al.*, 1984)
YFV (17D)	Vero	25	(Leyssen *et al.*, 2000)
DENV type-2	Vero	98	(Leyssen *et al.*, 2000)
YFV (17D)	Vero	6.2	(Neyts *et al.*, 1996)

that the compound could be viewed as a guanosine analogue (Prusiner and Sundaralingam, 1973). As discussed previously, ribavirin 5′-monophosphate (RTP) inhibits inosine-monophosphate dehydrogenase (IMPDH). The observation that mycophenolic acid and VX-497, compounds that solely inhibit IMPDH, are more potent inhibitors of flavivirus replication than ribavirin (our unpublished observations) (Markland *et al.*, 2000), points to the importance of inhibition of IMPDH, and thus GTP depletion, in the inhibition of flavivirus replication. However, it has been suggested that the depletion of intracellular GTP pools does not strictly correlate with antiviral activity (Smith, 1984). Several other potential mechanisms of antiviral action of ribavirin (against viruses other than flaviviruses) have been suggested. It has been shown that ribavirin 5′-triphosphate is able to competitively inhibit the guanylyltransferase activity of vaccinia virus (Goswami *et al.*, 1979). This observation can possibly be correlated with the observation that ribavirin-resistant Sindbis viruses could be generated that carry mutations in the guanylyltransferase gene (Scheidel and Stollar, 1991; Scheidel *et al.*, 1987).

Another target of ribavirin may be the viral RNA-dependent RNA polymerase (RdRp). RTP was shown to inhibit the RdRp of influenza virus (Erikson *et al.*, 1977; Wray *et al.*, 1985), La Crosse virus (Toltzis *et al.*, 1988), and vesicular stomatitis virus (VSV) (Toltzis and Huang, 1986; Toltzis *et al.*, 1988). There are also reports that suggest that ribavirin is capable of inhibiting the viral helicase/NTPase. In the case of reoviruses, RTP binds to a site close to the catalytic site of the transcriptase, which, by means of a conformational change, results in inhibition of the helicase activity (Rankin *et al.*, 1989). The binding of RTP would lower the affinity of the transcriptase for the template RNA so that the likelihood of premature termination would be greatly increased (Rankin *et al.*, 1989). The helicase of the hepatitis C virus was shown to be inhibited *in vitro* by RTP (Borowski *et al.*, 2000). The NTPase site of the helicase was blocked by RTP, thus arresting ATP hydrolysis. The energy that is generated during the hydrolysis of ATP is necessary for the unwinding of dsRNA. By blocking this process, helicase activity is also abolished.

Recently, it was revealed that ribavirin may also function as an RNA virus mutagen (Crotty *et al.*, 2000, 2001). This mechanism was first observed when studying the effect of ribavirin on poliovirus replication. It was shown that in the presence of ribavirin (at rather high concentrations: i.e., 400 and 1000 μM) the amount of incorporated mismatches or mutations in the viral genome increased 9.7 fold (Crotty *et al.*, 2001). This increase was attributed to the incorporation

of ribavirin in the viral genome and the ability of ribavirin to base pair equivalently with cytidine and uridine as long as the rotation of the carboxamide moiety was not impaired (Crotty *et al.*, 2000). The increase in mutation frequency was suggested to result in an "error catastrophe." As a result, the normal virus population, or quasispecies, shifted to a mutagenized population in which the majority of the viral genomes are lethally mutated (Crotty *et al.*, 2001). Recently, this mechanism was suggested to be applicable to two members of the *Flaviviridae*, in particular HCV (Maag *et al.*, 2001) and HGV-B (Lanford *et al.*, 2001).

Ribavirin is also believed to exert an immunomodulatory effect (Tam, 1999). Upon recognition of antigens, mature $CD4^+$ and $CD8^+$ T cells are induced to differentiate into two distinct functional subsets, i.e., type 1 (Th1) and type 2 (Th2), respectively. These subsets are distinguished according to the array of cytokines they produce. Type 1 cells are involved in T cell–mediated immune responses, such as defense against viruses, whereas type 2 cells assist B cells in regulating antibody production. The differentiation in type 1 and type 2 responses is strictly regulated. In concentrations substantially below the antiproliferative concentration, ribavirin is able to influence these regulatory mechanisms (Tam, 1999). Ribavirin enhances the Th1 response and diminishes the Th2 response by selectively altering type 1 and type 2 cytokine expression in T cells (Tam *et al.*, 1999a, 1999b). Ribavirin has also been shown to inhibit the production of pro-inflammatory mediators from virally activated macrophages (Ning *et al.*, 1998). However, it is questionable whether this immunomodulatory activity is an important mechanism of action of ribavirin. In this context, it is important to note that also the L-enantiomer of ribavirin (ICN 17261) exhibits immunomodulatory activity, but this compound has reduced antiviral effect (Tam *et al.*, 2000).

Despite the multitude of mechanisms that have been proposed for the antiviral activity of ribavirin, it remains unclear to what extent these mechanisms contribute to its (rather weak) anti-flavivirus activity. Likely, ribavirin exerts its action by a combination of the mechanisms mentioned above.

4. Activity of Ribavirin and its Derivatives against Flaviviruses

In vitro, ribavirin exerts weak to moderate activity against a variety of flaviviruses. An overview of the 50% effective concentrations (EC_{50}), as obtained in different studies, is given in Table II. EC_{50} values range from 8 to 250 μg/mL and the potency of ribavirin appears to be cell line–dependent. In general, ribavirin, in its unmodified form, has

proven to be ineffective in the treatment of flavivirus infections in animal models. It elicited no significant effect on viremia, morbidity and mortality in a rhesus monkey model for DENV infections (Malinoski *et al.*, 1990). Also, in the Modoc virus model, no protective effects were noted for ribavirin (Leyssen *et al.*, 2001). The lack of *in vivo* antiviral activity can most likely be attributed to (i) the relatively weak anti-flavivirus activity, (ii) the suboptimal oral bioavailability, and (iii) in case of encephalitis, the inability to pass the blood-brain barrier. A number of prodrug strategies have been explored in an attempt to increase the antiviral activity of ribavirin (Prokai *et al.*, 2000). These strategies consisted of making ribavirin either more lipophilic or to target ribavirin to specific organs such as the brain or liver. The lipophilic derivative, ribavirin-2′,3′,5′-triacetate, proved to be more active as compared to ribavirin in mice inoculated intracranially with DENV (Koff *et al.*, 1983). Another prodrug consists of ribavirin modified with a dihydropyridine unit that serves as a redox "targeter" and which is covalently linked to an OH-group of ribavirin (Canonico *et al.*, 1988). In untreated C57BL/6 mice infected with JEV, 100% mortality was observed, whereas 40–50% of the mice survived when they where treated with the latter prodrug. Another interesting prodrug of ribavirin may be the lactosaminated poly-L-lysine derivative (Di Stefano *et al.*, 1997). Use of this prodrug not only reduced the toxicity of ribavirin to red blood cells, but also targeted the drug toward the liver (Di Stefano *et al.*, 1997; Fiume *et al.*, 1998). The latter observation may be important for the treatment of infections with hepatotropic flaviviruses such as YFV.

Although many derivatives of ribavirin have been synthesized, only few appear to be interesting. These include EICAR (5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide) (Balzarini *et al.*, 1993; De Clercq *et al.*, 1991a), selenazofurin (2-β-D-ribofuranosylselenazole-4-carboxamide) (Srivastava and Robins, 1983) and tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide) (Srivastava *et al.*, 1977). The main mechanism of anti-flavivirus activity of these ribavirin derivatives is most likely mediated through inhibition of IMPDH. Tiazofurin and selenazofurin are metabolized into nicotinamide adenine dinucleotide (NAD^+)-analogues, which bind to the NAD^+-binding pocket of IMPDH. This results in depletion of cellular GTP pools (Cooney *et al.*, 1982, 1983; Gebeyehu *et al.*, 1985; Jayaram *et al.*, 1983). Also EICAR-5′-MP strongly inhibits IMPDH (Balzarini *et al.*, 1998) and the NAD-analogue of EICAR accumulates in the cell. It remains to be studied whether also this metabolite inhibits IMPDH (Balzarini *et al.*, 1998). *In vitro*, ribavirin and selenazofurin, as well as ribavirin and tiazofurin,

synergistically inhibit JEV and YFV replication, whereas the combination of selenazofurin and tiazofurin is additive against JEV (Huggins *et al.*, 1984). No reports have appeared on the treatment of infections with flaviviruses *in vivo* with these compounds.

5. Conclusion

Although ribavirin is widely used in combination with interferon for the treatment of chronic HCV infections, the compound has limited activity against flaviviruses. More insight in the precise mechanism of anti-flavivirus activity of ribavirin may possibly lead to the design of more potent and selective analogues. Also, studies on novel prodrug strategies may be of interest to increase the oral bioavailability, organ tropism, and blood-brain barrier penetration.

B. Interferon and Interferon Inducers

1. Introduction

Upon infection of the host, many cytokines are synthesized that play an important role in the host defense against viral pathogens. Interferon (IFN)-α/β (type I interferon), IFN-γ (a type II interferon) and tumor necrosis factor (TNF)-α have the potential to trigger the activation of intracellular antiviral pathways after they bind to specific receptors on the surface of infected cells, or to protect the cells from being infected by inducing antiviral defense mechanisms (reviewed by Guidotti and Chisari, 2000). Other cytokines, or immunomodulating compounds, may contribute indirectly to the antiviral response by upregulating or inducing the chemokines mentioned above. IFN-α/β and IFN-γ are produced by different cell types and interact with different cellular receptors, resulting in the activation of different intracellular signaling pathways (reviewed by Guidotti and Chisari, 2000).

Predominantly leukocytes, but also many other cell types, produce IFN-α in response to a viral infection. Following binding of IFN-α to its receptor on the surface of a noninfected cell, intracellular tyrosine kinases, belonging to the Janus kinases, are phosphorylated and activated. An intracellular signal transduction cascade is initiated, which involves several STAT proteins (signal transducers and activators of transcription). Upon activation, they dimerize and move into the nucleus to interact with responsive elements on the host cell DNA. Genes are expressed, leading to the production of host cell proteins, such as cytoplasmic RNases, that would inhibit intracellular viral replication upon infection.

IFN-β, which is mainly produced by fibroblasts, upregulates the production of major histocompatibility complex (MHC) class I antigens, which are expressed by all host cells and which present foreign antigens of intracellular origin (such as viral proteins, produced upon infection of the host cell) to the immune cells, thereby increasing the likelihood that a virus-infected host cell is recognized by the immune system. Furthermore, IFN-β also stimulates the activity of natural killer cells that are involved in the elimination of virus-infected cells.

Although IFN-γ also possesses some antiviral properties, it has mainly an immunomodulatory function. IFN-γ, together with cytokines of the Th1 subset, such as TNF-β and IL-2, play an important role in the activation and regulation of the cellular immune response, which requires macrophage and T-cell activation. Furthermore, it also mediates the humoral immune response by inducing antibody production of the IgG2a subtype.

TNF-α mediates selective host cell destruction by direct cell-to-cell contacts as part of the cellular immune response (Kuby, 1994; www.copewithcytokines.de/). From this, one may thus conclude that in particular several subtypes of interferon α may show promise for the therapy of viral infections. This has been confirmed by their use for the treatment of infections with for example the hepatitis C virus.

2. Interferon

In cell culture, IFN-α and IFN-β are able to protect cells against infection with DENV. The effect of IFN-γ on DENV infection proved to be more variable (Diamond *et al.*, 2000). The recombinant consensus interferon YM643 (interferon alfacon-1) was more inhibitory than IFN-α against YFV infection in cell culture (Yasuda *et al.*, 2000).

In mice, IFN-α and IFN-β demonstrated some therapeutical benefit against Banzi virus infection, whereas IFN-γ was only protective when treatment was initiated before the infection (Pinto *et al.*, 1988, 1990). When IFN-γ was administered before and after infection of monkeys with YFV, a delay in disease progression and even some transient improvement was observed, although ultimately no change in overall mortality was noted (Arroyo *et al.*, 1988). Prophylactic, but also therapeutic, treatment with IFN-α significantly reduced mortality in mice infected with SLEV (Brooks and Phillpotts, 1999).

In 1981, Cuba started a program for the large-scale production of IFN-α (www.cariscience.org/csarticle01.htm, www.cubasolidarity.net/waitzkin.html). That year, IFN-α was used during a serious epidemic of dengue fever in Cuba itself. Amelioration of disease following intramuscular administration of interferon was reported in children and

adults with primary DENV serotype 2 infection (Limonta *et al.*, 1984). These results have, however, never been confirmed. In a small clinical trial in which two patients with severe comatous symptoms following Japanese encephalitis virus infection were treated with human recombinant IFN-α, quick improvement of the general condition and complete recovery from the comatous stage were reported (Harinasuta *et al.*, 1985).

3. Interferon Inducers

Interferon inducers also proved to be active against flavivirus infections in animals. Poly(inosinic acid)-poly(cytidylic acid) [Poly(IC)], a synthetic double-stranded RNA and a potent inducer of IFN-α/β, delayed Modoc virus (MODV)–induced morbidity and mortality in SCID mice (Leyssen *et al.*, 2001). Poly (IC) was also previously reported to cause protective activity against experimental infection of mice with tick-borne encephalitis virus (Kunz and Hofmann, 1969), Japanese encephalitis virus (Harrington *et al.*, 1977; Singh and Postic, 1970), and West Nile virus (Haahr, 1971). Ampligen, [poly (I)-poly ($C_{12}U$), which is poly(IC) with a U mismatch at every 12th base of the C strand], was found to protect mice against lethal Banzi virus infection (Pinto *et al.*, 1988). Other interferon inducers or immunomodulators, such as tilorone hydrochloride [2,7-bis-2-(diethylamino)-ethoxy-fluoren-9-onedihydrochloride] (Hofmann and Kunz, 1972; Vargin *et al.*, 1977), 6-MFA (Ghosh *et al.*, 1984, 1990), carboxymethylacridanone (Taylor *et al.*, 1980), CL246,738 [3,6-bis(2-piperidino-ethoxy)acridine trihydrochloride] (Pinto *et al.*, 1988; Sarzotti *et al.*, 1989), and poly(ICLC) [a poly(IC), poly-L-lysine, carboxymethyl cellulose mixture] (Singh *et al.*, 1989; Stephen *et al.*, 1997) were found to elicit some protective effect against various flavivirus infections in animals.

4. Treatment of Hepatitis C Virus Infections with Interferon

Currently, chronic and early diagnosed acute infections caused by HCV are treated with IFNα-2 alone (with a sustained virologic response in 15–20% of the patients) (Lau *et al.*, 1998) or in combination with ribavirin (which upgrades this sustained response to about 40%) (Davis, 1999; Davis *et al.*, 1998). The interferons approved for anti-HCV therapy are interferon α-2b (Intron-A), interferon α-2a (Roferon-A), consensus interferon (r-metIFN-Con1), and interferon α-1n (Weveron). All appear to be clinically equivalent (Gish, 1999; as reviewed by Leyssen *et al.*, 2000). Pegylation is considered to increase the relatively short half-life of IFN-α within the body, to reduce its adverse effects, and to avoid repeated "peak-trough cycling," which is believed to contribute

to drug intolerance during IFN-α therapy (Harris *et al.*, 2001; Kozlowski *et al.*, 2001). Initial data on the benefit of treatment with pegylated interferon α-2b, as compared to interferon α-2b, in patients with compensated chronic HCV infection has been reported (Fried *et al.*, 2002; Lindsay *et al.*, 2001). Peginterferon plus ribavirin is now the standard recommendation based on an NIH consensus statement.

5. *Conclusions*

Although therapy with interferon (or interferon inducers) may initially seem to be a valuable option for the treatment of infections with flaviviruses, diagnosis of the infection before the onset of symptoms is essential to warrant such therapy. The beneficial effect of interferon and interferon inducers was only observed when treatment was initiated before or very shortly following experimental infection. At the time that severe symptoms appear in the clinical setting, fever has abated in most cases and viremia is low or undetectable. At that time, it may be too late to recommend antiviral therapy with interferon. In addition, severe disease, deterioration, and death are by and large caused by immune-related mechanisms, which are triggered by the viral infection (e.g., in dengue hemorrhagic fever) (Chaturvedi *et al.*, 2000), and which may be adversely influenced by interferon, particularly in such cases.

C. *Miscellaneous*

The antiviral activity of several compounds against the replication of flaviviruses has been reported. The agents that have been used are listed in Table III, together with the class of molecules to which they belong, the flavivirus(es) against which they have been assessed, and the model(s) in which they have been assayed.

As discussed previously, several molecules that alter the nucleoside/nucleotide metabolism have a significant impact on virus replication, because they may lower the intracellular concentration of necessary building blocks such as CTP and UTP. Cyclopentenylcytosine (Marquez *et al.*, 1988), an inhibitor of CTP synthetase, was shown to be effective *in vitro* against vaccine strains of YFV and JEV (both assays in Vero cells). Similarly, inhibitors of OMP-decarboxylase such as 6-azauridine and pyrazofurin have been found to markedly inhibit flavivirus replication (Neyts *et al.*, 1996).

Other compounds that affect cellular processes and that have been reported to inhibit the replication of flaviviruses are castanospermine and deoxynojirimycin (Courageot *et al.*, 2000). Both are α-glucosidase inhibitors, which strongly affect the productive folding pathways of the

TABLE III

ANTI-FLAVIVIRUS ACTIVITY OF MISCELLANEOUS AGENTS

Compound	Class	Virus	Results	References
Cyclopentenylcytosine	CTP synthetase inhibitor	JEV	$EC_{50} = 0{,}1$ μg/mL in Vero cells	(Marquez *et al.*, 1988; Neyts *et al.*, 1996)
Pyrazofurin	OMP decarboxylase	YFV	$EC_{50} = 0{,}37 \pm 0{,}18$ μg/mL in Vero cells	(Neyts *et al.*, 1996)
6-azauridine	OMP decarboxylase inhibitor	YFV	$EC_{50} = 0{,}41 \pm 0{,}07$ μg/mL in Vero cells	(Neyts *et al.*, 1996)
Amantadine hydrochloride	Influenza M_2 channel blocker	DENV	Weak activity *in vitro* at subtoxic concentrations	(Koff *et al.*, 1980)
Guanine 7-N-oxide	—	JEV	$EC_{50} = 30 \pm 5$ μg/mL in BHK cells	(Yamamoto *et al.*, 1990)
Polyanions	Inhibitors of virus adsorption	DENV YFV	EC_{50} ranging from 9 to 27 μg/mL against YFV in Vero cells	(Marks *et al.*, 2001; Neyts *et al.*, 1996)
Castanospermine and deoxynojirimycin	α-glucosidase inhibitors	DENV	Neuro2a cells	(Courageot *et al.*, 2000)
Brefeldin A	Fungal metabolite	WNV	Vero cells	(Sreenivasan *et al.*, 1993)
Furanonaphtoquinone derivatives	—	JEV	$EC_{50} \approx 0{,}1$ μg/mL in BHK cells	(Takegami *et al.*, 1998)
Dehydroepian-drosterone	Corticosteroid hormone	WNV	40–50% mortality as compared to 100% in untreated CDR-1, BR mice	(Ben-Nathan *et al.*, 1991; Ben-Nathan *et al.*, 1992)
Melatonine	Neurohormone	Attenuated WNV	25–31% mortality as compared to 50–75% in untreated ICR (CD1) mice	(Ben-Nathan *et al.*, 1995)
Amaryllidaceae isoquinoline alkaloids	Alkaloids	JEV, YFV, DENV	*In vitro* activity in LLC-MK2 cells at subtoxic concentrations 0–20% mortality as compared to 100% in untreated C57B1/6 mice	(Gabrielsen *et al.*, 1992)
Astragali radix extracts	Plant extracts	JEV	10–50% mortality as compared to 95% in untreated ICR mice	(Kajimura *et al.*, 1996)

envelope glycoproteins prM and E, and thus prevent viral particles from assembling. Another compound that interacts with the glycosylation pathway is brefeldin A (BFA) (Sreenivasan *et al.*, 1993). This molecule displayed some activity on WNV replication in Vero but not in C6/36 (mosquito) cells. BFA may be a useful tool in the study of flavivirus glycoprotein processing, but may not be attractive as an antiviral agent.

Amantadine hydrochloride, an influenza A M_2 channel blocker, was reported to confer some, albeit very weak, *in vitro* antiviral activity against DENV-2 (Koff *et al.*, 1980). Guanine 7-N-oxide, isolated from *Streptomyces* sp., elicited antiviral activity against JEV in BHK cells ($IC_{50} = 30 \pm 5$ μg/mL) (Yamamoto *et al.*, 1990). The selectivity index, which was calculated based on the cytostatic effect, yielded, however, a value <1, although guanine-7-oxide proved to be not cytotoxic to confluent BHK cells.

A protective effect (i.e., a 40–50% reduction in overall mortality) of dehydroepiandrosterone on WNV-associated mortality in mice was observed (Ben-Nathan *et al.*, 1991, 1992). The mechanism underlying this protection is believed to reside in an effect on host resistance mechanisms rather than virus replication *per se*. In ICR mice inoculated with an attenuated strain of WNV (which only causes encephalitis under stress conditions), melatonin was reported to delay (within 7–10 days) and to reduce WNV-associated mortality (Ben-Nathan *et al.*, 1995). However, to be able to achieve this protection, treatment with melatonin had to be initiated 3 days before infection.

Several polyanionic compounds have been shown to prevent enveloped viruses from adsorbing to the host cell (Marks *et al.*, 2001). Heparin, heparin-derived oligosaccharides, and the polysulfonate suramin inhibited DENV envelope protein (E) binding to the host cell surface in a competition assay. Dextran sulphate (MW 5000) and PAVAS (the co-polymer of acrylic acid with vinyl alcohol sulphate) prevented YFV from attaching to Vero cells at an EC_{50} value of 9–27 μg/mL (Neyts *et al.*, 1996).

Isoquinoline alkaloids derived from *Amaryllidaceae* inhibited JEV replication *in vitro*, albeit with a low potency and selectivity (Gabrielsen *et al.*, 1992). Yet, these compounds were shown to increase the survival of JEV-infected mice by 80–100%. Extracts from *Astragali radix* (Kajimura *et al.*, 1996) were shown to decrease JEV-induced mortality in mice by 60–80%, although no protective effect was observed in cell culture.

Furanonaphtoquinone derivatives (Takegami *et al.*, 1998), in particular FNQ3 [2-methyl-naphto(2,3-b)furan-4,9-dione], showed a

dose-dependent inhibition of JEV yield in Vero cells. In the presence of 10 μg/mL FNQ3, virus yields decreased to 2% of the control. It was suggested that FNQ3 inhibits JEV replication by interfering with viral RNA and protein synthesis. Macrophage-derived neutrophil chemotactic factor (MDF) delayed JEV associated mortality, but had no overall effect on the survival of JEV-infected mice (Saxena *et al.*, 2000, 2001). The mechanism of this protective effect may be mediated by NO.

IV. Animal Models

Most human flaviviruses require special research facilities—for example, bio-safety level 3 (BSL3) conditions for manipulation of yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), louping ill virus (LIV), Murray Valley encephalitis virus (MVEV), Powassan virus (POW), and even BSL4 containment facilities for tick-borne encephalitis viruses such as Russian spring-summer encephalitis virus (RSSEV) and Omsk hemorrhagic fever virus (OHFV) (www.cdc.gov/od/ohs/biosfty/bmbl/sect7f.htm). Ideally, a virus used in a model for the evaluation of novel antiviral strategies should (i) cause morbidity and mortality in small (adult) laboratory animals following systemic inoculation, (ii) not be pathogenic to humans, and (iii) mimic human disease. After a compound would prove to be active against a selection of flaviviruses in cell culture, the prophylactic or therapeutic value should be assessed in an experimental rodent model. If activity is observed in murine or hamster models, efficacy studies may be performed in monkey models, and the activity of the compounds evaluated against more pathogenic human flaviviruses.

Several flaviviruses that cause infections in mice have already been employed to assess the potential efficacy of selected molecules. The Banzi virus (BZV)(BSL2) is lethal to immunocompetent mice following intraperitoneal inoculation (Pinto *et al.*, 1988; Singh *et al.*, 1989; Smee *et al.*, 1987), the Langat virus (LGTV)(BSL2) causes a lethal infection after inoculation by the intraperitoneal and intracerebral route (Vargin *et al.*, 1977), whereas dengue virus (DENV)(BSL2) infection resulted in severe disease only when inoculated directly into the brain (Koff *et al.*, 1983; Vargin *et al.*, 1977). Also the Modoc virus (MODV) (BSL2) may be of particular interest. SCID mice inoculated via the intraperitoneal route develop lethal encephalitis. Immunocompetent hamsters infected systemically with MODV shed the virus in the urine, which allows evaluation of antiviral strategies for a long time without using invasive sampling methods and allows the viral titers

in the urine of individual animals to be monitored (Leyssen *et al.*, 2001).

Because of the higher biosafety precautions, only limited use has been made of mice infected with WNV (BSL3) (Ben-Nathan *et al.*, 1991, 1992, 1995; Vargin *et al.*, 1977), or JEV (BSL3) (Kajimura *et al.*, 1996), and tick-borne encephalitis virus (TBEV) (BSL4) (Gresikova *et al.*, 1982), as a model for the study of therapies for the treatment of flavivirus infection.

In only few studies, antiviral effects were assessed against YFV (BSL3) (Huggins, 1989), DENV (BSL2) (Huggins, 1989; Malinoski *et al.*, 1990), or JEV (BSL3) infections in monkeys (Ghosh *et al.*, 1984, 1990).

Other models for flavivirus infection in mice have been developed to investigate the protective effect of experimental vaccines, or to develop a model for the study of the pathology and pathogenesis of the infection. SCID mice reconstituted with human peripheral blood lymphocytes and infected subsequently by the intraperitoneal route with DENV (type 1), showed viremia, although virus production was highly variable (Wu *et al.*, 1995). SCID mice engrafted in the peritoneal cavity with human erythroleukemia cells (K562) and infected with DENV (type 2) in the resulting abdominal tumor mass five weeks after implantation, developed encephalitis and died. DENV replication was recorded in the serum, the brain, and the tumor mass (Lin *et al.*, 1998). Infection of SCID mice transplanted with HepG2 cells yielded similar results (An *et al.*, 1999). Mice that lack the α/β and γ interferon and receptor genes (AG129 mice) developed disease and died 10–12 days after intraperitoneal inoculation with a mouse-adapted type 2 DENV. In contrast to wild-type or mice deficient in either the α/β interferon receptor or γ interferon ligand genes, replication of the virus could be demonstrated in the sera, spleens, and brains of the infected mice (Johnson and Roehrig, 1999). Infection with JEV of mice younger than 3 weeks by the intracerebral or peripheral route resulted, depending on the strain used, in encephalitis and death (Higgs and Gould, 1991). Wild-type YFV infection in hamsters induced disease that resembled the clinical and pathological characteristics observed in humans (Tesh *et al.*, 2001; Xiao *et al.*, 2001). The YFV 17D strain, which is currently being used as the vaccine strain, may be adapted to become neurovirulent and neuroinvasive after serial passage in mice (Chambers and Nickells, 2001). Similarly, inoculation of TBEV, adapted to become neurovirulent in mice and to be used as a live-attenuated vaccine (Venugopal and Gould, 1994), causes encephalitis and death in suckling or young adult mice (Holzmann *et al.*, 1990; Kaluzova *et al.*, 1994). Furthermore, the Langat virus (LGTV), which

is a naturally attenuated tick-borne encephalitis virus, is neuroinvasive and neurovirulent for SCID mice (Pletnev, 2001). These and other models may be of utility in the investigation of the antiviral activity of novel compounds.

V. Perspectives

Despite the major clinical impact of flaviviruses, there is as yet no drug available for the chemoprophylaxis or chemotherapy of infections with these viruses in humans. The intensive search for inhibitors of the replication of HCV will hopefully result in the discovery of compounds that inhibit the replication of flaviviruses at large.

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