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C. J. Peters and C. H. Calisher (eds.)

Infectious Diseases from Nature: Mechanisms of Viral Emergence and Persistence

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Preface

It is rare for a remarkable human being to come into one's life. Dr. Robert E. Shope, M.D., a truly remarkable human being, entered the lives of hundreds or thousands, made their lives better, served as an example of the best of humanity, accomplished a huge amount professionally, and did all this and much more with equanimity, charm and intellect. At any scientific gathering, several people, often from far-flung areas of the world, would approach someone from Bob's institution for the simple purpose of sending regards, a phenomenon rarely seen with anyone else in our experience. Bob was neither manipulative nor confrontational but convinced people by the force of his knowledge and personality. He brought the virus community together and served as a bridge between classical arbovirology and many other disciplines. He was responsible for applying genetics, structural biology, ecology, climate, and many other facets of arbovirology, although he was not personally expert in any of those fields. When things needed to be accomplished and he saw no one else would act, he would take it upon himself to do so.

In November 2003, as a tribute to Bob, the University of Texas Medical Branch at Galveston dedicated its new biosafety level four laboratory to him. A grand ceremony was held and the Robert E. Shope Laboratory officially opened. In addition, a symposium to honor Bob was planned for March 18–21, 2004. Unfortunately, Bob died in January 2004, succumbing to the effects of a chronic illness, so the purpose of the symposium was changed to that of a memorial.

The symposium was held at the San Luis Resort and Conference Center on Galveston Island and was the Third James W. McLaughlin Foundation Symposium on Infectious Diseases. This symposium brought together many outstanding virologists from around the world, people who knew Bob for many years, who had worked with him, who admired him and who genuinely liked him. It was clear from the public comments of many of them that they had been deeply touched by Bob and already missed him. It is unimaginable to us that we will no longer see his smiling face, have his sage advice, benefit from his encyclopedic knowledge, or have his leadership. Still, as with all great people, Bob Shope left a remarkable legacy, including numerous and varied contributions to the scientific record.

The papers included in this Special Issue of Archives of Virology were presented at the March symposium by those who accepted our request to prepare written remarks for a published memorial to Bob. The title, "Infectious Diseases from Nature: Mechanisms of Viral Emergence and Persistence", reflects the focus of much of Bob's professional efforts and these papers address a wide-ranging

spectrum of topics that would have interested him and to which he had contributed in one fashion or another. We think he would have loved it.

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Comments on the life and contributions of Robert E. Shope

R. B. Tesh

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I first met Bob Shope in 1965 in Brazil. At the time he was Director of the Rockefeller Foundation's Virus Laboratory at the Evandro Chagas Institute in Belem, and I was a young Peace Corps physician stationed in Recife. We remained friends for almost 40 years, and we worked together at the University of Texas Medical Branch (UTMB) and at Yale for 25 years. Although most of us knew Bob as a virologist, teacher, loyal colleague and friend, he was also a world-class epidemiologist.

Perhaps because of his training as a physician, Bob maintained an interest in disease causality and disease transmission. He believed deeply in the importance of public health. For 30 years, he was a member of the Epidemiology Section of the Yale School of Public Health. At Yale University, the School of Public Health is a department within the School of Medicine; and Bob often told me that the University had it backwards and that Schools of Medicine should be within Schools of Public Health. He firmly believed that, and I think he was probably correct.

Bob was truly a citizen of the world, being equally at home in Beijing, Riyadh, Geneva, Washington, Belem or anywhere else. He was the same guy wherever he went. And he was deeply committed to global public health. He was a frequent consultant to the World Health Organization (WHO) and to the PanAmerican Health Organization (PAHO), as well as to many foreign governments, in the areas of vector-borne and zoonotic viral diseases of public health importance. For about 15 years he was a member of the WHO Expert Committee that oversaw field trials of vaccines for dengue and Japanese encephalitis in Thailand; for several years, he chaired that Committee. Bob also was a long-time member of PAHO's Expert Committee on Yellow Fever and Dengue in the Americas. He was a member of the Armed Forces Epidemiological Board and a frequent consultant to the Fogarty Institute at NIH, the Department of Defense, the Institute of Medicine, National Research Council, U.S. Department of Agriculture, the Baker Institute at Cornell University, and various foundations. He had broad interests and a unique ability to switch between subjects such as viral diseases, vaccine strategies,

animal health, and the effects of global climate change on human health. He was widely published. Bob was co-editor, along with Joshua Lederberg, of the seminal publication, "Emerging Infections: Microbial Threats to Health in the United States". He also was a frequent contributor to textbooks on infectious diseases, virology, medicine and public health. At UTMB, Bob had a joint appointment in the Department of Preventive Medicine and Community Health, and he lectured regularly there to graduate students, residents and fellows.

I believe that Bob's important contributions to public health often were overshadowed by his accomplishments in other areas, and were not fully appreciated. The field of public health never seems to receive the political or media attention that the newest emerging disease receives.

It is very appropriate that the newly designated Professorship in his honor at UTMB will be supported jointly by the Departments of Pathology and of Preventive Medicine and Community Health. Bob believed deeply in public health, and he was always an advocate for its inclusion within medical training and practice. In addition, the American Society for Tropical Medicine and Hygiene has established the Robert E. Shope, M.D. Fellowship in Infectious Diseases, which will provide training for promising clinicians, epidemiologists, and virologists. The legacy Bob has left does not fill the hole in our lives caused by his death, but at least his footprints remain.

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Virus perpetuation in populations: biological variables that determine persistence or eradication

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Summary. In this review, I use the term "perpetuation" for persistence of a virus in a population, since this is a different phenomenon from persistence of a virus in an infected host. Important variables that influence perpetuation differ in small (<1,000 individuals) and large (>10,000) populations: in small populations, two important variables are persistence in individuals, and turnover of the population, while in large populations important variables are transmissibility, generation time, and seasonality. In small populations, viruses such as poliovirus that cause acute infections cannot readily be perpetuated, in contrast to viruses such as hepatitis B virus, that cause persistent infections. However, small animal populations can turnover significantly each year, permitting the perpetuation of some viruses that cause acute infections. Large populations of humans are necessary for the perpetuation of acute viruses; for instance, measles required a population of 500,000 for perpetuation in the pre-measles vaccine era. Furthermore, if an acute virus, such as poliovirus, exhibits marked seasonality in large populations, then it may disappear during the seasonal trough, even in the presence of a large number of susceptible persons. Eradication is the converse of perpetuation and can be used as a definitive approach to the control of a viral disease, as in the instance of smallpox. Therefore, the requirements for perpetuation have significant implications for practical public health goals.

Introduction

From the viewpoint of the individual host, viral infections can be conveniently divided into those that are acute and those that are persistent. However, all viruses – by definition – must be able to persist in their host population, regardless of whether they cause acute or persistent infection in individual members of that population. Thus, persistence in a population is a distinct phenomenon and in this discussion I will use the term "perpetuation" to distinguish it from persistence in the individual host.

Table 1. Biological parameters that influence perpetuation of a virus in a host population. Based in part on [30][a]

Parameter	Small population <1,000	Large population >10,000
Persistence in the individual host	++++	
Population turnover	++++	
Transmissibility and generation time		++++
Seasonality		++++

[a] ++++: particularly important parameter

Once a virus has infected a defined population, it may either perpetuate indefinitely or may disappear. If disappearance is a natural occurrence, it is often described as "burn out" or "fade out", while if it is induced by human intervention, it may be described as "eradication" or "elimination". Eradication represents a definitive approach to prevention of a viral disease, as in the instance of smallpox. However, to develop a strategy for eradication it is necessary first to understand the requirements for perpetuation. Thus, the subject has significant implications for practical public health goals.

Virus persistence and perpetuation has been the subject of numerous discussions, and this presentation draws heavily on some of these publications [1, 26, 30]. Some of the biological variables that influence perpetuation are shown in Table 1. Implicit in this table is the generality that most viruses can infect a given host only once. In the instance of an acute infection, the host acquires lifelong immunity to the infecting virus and is – from an epidemiological perspective – no longer capable of acting as a link in the chain of infection. If the virus causes persistent infection, then the outcome varies. Some persistent virus infections can be transmitted as long as the host is infected (for instance hepatitis B virus and human immunodeficiency virus [HIV]). Other viruses (such as varicella zoster and herpes simplex) persist in a latent form and are infectious only during intermittent episodes of recrudescence.

Virus perpetuation within a human population involves a fragile equilibrium between three different categories of hosts: those who have not been infected and are susceptible; those who are actively infected and are potentially infectious; and those who have been infected and are immune. If the infection spreads too slowly within the population (transmissibility quotient, $Ro < 1$) the virus will ultimately disappear for absence of actively infected hosts. On the other hand, if the infection spreads too rapidly ($Ro \gg 1$), the susceptible population will be "exhausted", also leading to disappearance of actively infected hosts.

The size of the population under consideration is an important determinant of the dynamics of perpetuation, since the relative importance of other variables is different in smaller (<1,000 individuals) and larger (>10,000) groups (Table 1).

In small populations, two of the most important variables are persistence in the individual host and population turnover (the rate at which new susceptible animals are introduced into the population). In large populations, variables of high importance include transmissibility, generation time, and seasonality. Transmissibility (Ro) is the number of new infections that are generated by each existing infection and is a property (in part) of each virus, since under a given set of conditions, some viruses will be transmitted at a much higher rate than will others. Generation time is the average time between the infection of two individuals who are successive links in an infection chain; generation time may be a short as 2–3 days in the case of influenza and as long as many years in the case of HIV or hepatitis B infection. Seasonality refers to the variation in transmissibility of a given virus in a specific population at different times of year.

Perpetuation in small populations

Viruses that cause acute infections are often unable to perpetuate in small populations [3]. Figure 1 shows a seroepidemiological study of poliovirus in a small Eskimo village in Greenland, conducted in the 1950s. Each of the three types of poliovirus had been introduced into this population. Type 1 virus had caused an outbreak of infection 25 years prior to the study and had then disappeared; type

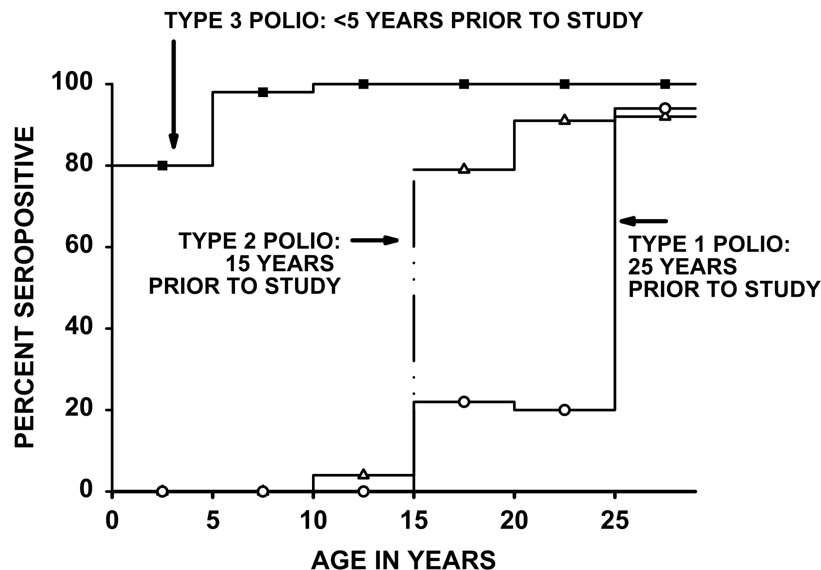

Fig. 1. Age distribution of poliovirus antibodies in an isolated Eskimo village, Narssak, Greenland. The data show three separate introductions of types 1, 2, and 3 poliovirus, respectively. The low frequency of type 1 antibodies in persons ages 15–25 probably represents cross-reacting antibodies induced by infection with type 2 virus. It appears that this acute infection "burned out" in this small (<1,000) isolated population because it spread rapidly through persons who had not been previously infected and "exhausted" the susceptible population. After [19]

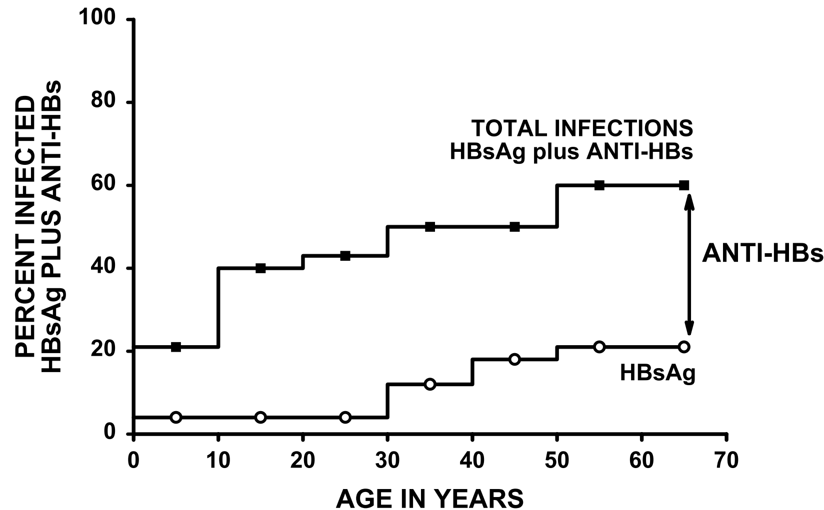

Fig. 2. Age distribution of hepatitis B surface antigen (*HbsAg*) and antibody to HbsAg (*anti-HBs*) in Eskimos of southwest Greenland. HBV was perpetuated in this small isolated population (<1,000) because it caused lifelong persistent infections in some persons (*HbsAg-positive*) who could continue to spread infection to susceptible newborn infants. Perpetuation was also enhanced by the low transmissibility of HBV, resulting in a pool of susceptible adults (persons who escaped infection as children and were infected as adults). After [25]

2 virus had been introduced 15 years prior to the study date and had likewise disappeared; and type 3 had been introduced within the prior 5 years and (likely) had also disappeared. In such small populations, viruses that cause acute infections spread so rapidly that they quickly exhaust the susceptible population and then fade out. Conversely, hepatitis B virus, which causes both acute and persistent infections can persist in small populations as shown in Fig. 2, a study of another small Eskimo population in Greenland. In such populations hepatitis B virus is often transmitted during birth, from infected mothers to their newborn infants, which frequently results in persistent infections.

Another parameter that favors virus perpetuation is rapid turnover of the population itself. This is seen most often in animal populations some of which, in nature, may have an average lifespan of 1–2 years, so that a large fraction of the population consists of relatively young and susceptible hosts. Although difficult to document in wildlife populations, this phenomenon can be more readily documented in groups of laboratory animals that are under constant surveillance. One example is a study conducted in a colony of laboratory rats that was maintained for nutritional studies [21]. This colony was infected with rat parvovirus, a small DNA virus that did not cause overt disease and was only detected by serological surveillance. Rat parvovirus caused an acute infection, transmitted by the enteric route, that spread rapidly through the relatively small population of about 500 young animals. Based on the rate of spread, the virus might have been expected to exhaust all susceptibles by 10 months of age. However, every month about 25% of the animals aged

4–5 months were removed to another room to be used for experiments and the same number of one-month susceptible weanling animals was introduced from a breeding colony. This continual introduction of young susceptible animals was sufficient to perpetuate an acute virus infection in a small population.

Perpetuation in large populations

As mentioned above, although a number of viruses cannot be maintained in small human populations, all human viruses are capable of perpetuation in large populations. Important biological determinants of perpetuation include transmissibility, generation time, and seasonality, and these three may, in turn, determine the minimum size of the population required for perpetuation. Transmissibility (Ro) reflects in part the innate infectivity of a given virus, but is also determined by the density of the population, by the proportion of that population that is susceptible, and by the frequency of significant contact between different individuals within the population. The following examples illustrate the interaction of all these variables, and indicate the complexity of these relationships.

Measles

Measles has a special place as an example of virus perpetuation, since it is a rare instance where public health statistics can be used to monitor the ebb and flow of a specific virus infection in large human populations. Measles has several attributes that – in the aggregate – are not seen for other common viral diseases: (i) There are longterm records of measles incidence, collected by many health departments in the United States and other countries; (ii) 95% of all measles infections manifest as illness (in contrast to 1% for poliomyelitis for example); (iii) the symptoms of measles are sufficiently pathognomonic so that it can be distinguished from other viral infections by clinical observers; and (iv) population-wide reports can be corrected for under-reporting (about 15% of measles cases were reported in most cities in the United States prior to the introduction of measles vaccine in 1963).

Exploiting these facts, Bartlett [2] published several classical studies showing that in the pre vaccine era in the United States, measles was perpetuated in cities of 500,000 or greater population but not in cities below that size. Similar observations could be made in other parts of the world. For instance, in Iceland, with a population of 150,000 to 200,000, measles was introduced about 6 times during the period 1900 to 1940; each time it caused an outbreak that lasted 1–3 years, and then disappeared (Tauxe, unpublished, 1979).

Although these data are striking, they remained unexplained for a number of years. Why was 500,000 the limiting population size, at least in the cities included in Bartlett's study? A putative explanation was put forth in several papers that focused on the seasonality of measles in temperate climates [30]. Data for Baltimore (one of the cities included in Bartlett's study), for the period 1928–1961, are shown in Fig. 3. Absent seasonality, 8% of annual incidence

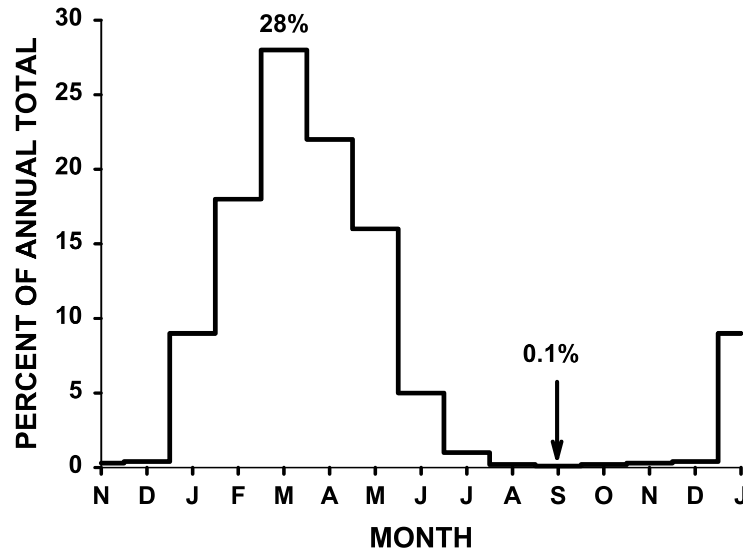

Fig. 3. The seasonality of measles in Baltimore, MD, 1928–1961, for 16 years of high incidence, showing the relative numbers of cases for each month. After [31]

Table 2. The number of measles cases during the trough period in a hypothetical North American city of 500,000 population, prior to the introduction of measles vaccine, based on data from [30][a]

Population	500,000
Measles susceptibles (estimated 10% of population)	50,000
Annual measles incidence (estimated average)	10,000
Cases in trough month (0.1%)	10
Cases in trough generation period (12 days)	3

[a]An age profile for measles susceptibles was constructed from the age distribution reported for measles in Baltimore, MD, for 1900–1931, supplemented with serosurveys conducted prior to the introduction of measles vaccine. The average number of annual measles infections was estimated as the size of an annual birth cohort, assuming a steady state and 100% cumulative attack rate for measles. Cases in trough month based on data from Baltimore, MD, 1928–1961, after [30]

would have been expected each month; however measles peaked in March at 28% while only 0.1% was reported in September, the trough month. Based on these observations, a hypothetical reconstruction for a city of 500,000 with 0.1% of measles in the trough month is shown in Table 2. In such a city, during a single trough generation period, only 3 cases of measles would be expected. Under these circumstances, it is plausible that measles infection could fade out.

A further test of the hypothesis that seasonality played a critical role in the fade out of measles is provided by data from New York City and Baltimore, prior to and after the introduction of measles vaccine (Table 3). The data in Table 2 imply

Table 3. The effect of measles immunization on the perpetuation of measles in a large population, after [30][a]

	Year	New York City		Baltimore	
		No. of susceptibles	Measles cases reported in the trough month	No. of susceptibles	Measles cases reported in the trough month
Pre vaccine	1958	900,000	47	90,000	14
	1959		97		22
	1960		43		11
	1961		123		19
Measles vaccine introduced	1963				
Post vaccine	1968	400,000	11	40,000	0
	1969		39		0
	1970		31		0
	1971		39		0

[a]The estimated number of susceptibles is based on the age distribution of measles cases and serosurveys of measles antibody, after [30]

that a population of about 50,000 susceptibles (data not shown indicate that about 10% of the total population was susceptible to measles) was required to perpetuate measles in cities of North America prior to the introduction of measles vaccine. In New York City, it can be estimated that there were about 900,000 susceptibles prior to measles vaccine and about 400,000 in the late 1960s, after the introduction of measles vaccine. As Table 3 shows, measles was perpetuated in New York City after the introduction of the vaccine. In Baltimore, vaccination was estimated to reduce the susceptible population from 90,000 to 40,000, just below the threshold for perpetuation. In fact, measles was perpetuated in Baltimore prior to measles vaccination, but showed an annual fade out each year in the late 1960s, after the introduction of measles immunization.

Poliomyelitis

Currently, the global effort to eradicate poliovirus is moving towards its goal. In 1988, when WHO enunciated the eradication of polio as a goal, there were an estimated annual 350,000 cases of paralytic poliomyelitis worldwide; in 2001, there were fewer than 1,000. As we approach eradication, it is interesting to look back at the origins of this effort, the eradication of wild poliovirus in the United States in 1972 (Fig. 4). Amazingly, although poliomyelitis was being tracked carefully by the Centers for Disease Control and other public health specialists, no one anticipated eradication of wild poliovirus [16]. The explanation for this apparent paradox is not hard to find. Public health surveillance was focused on

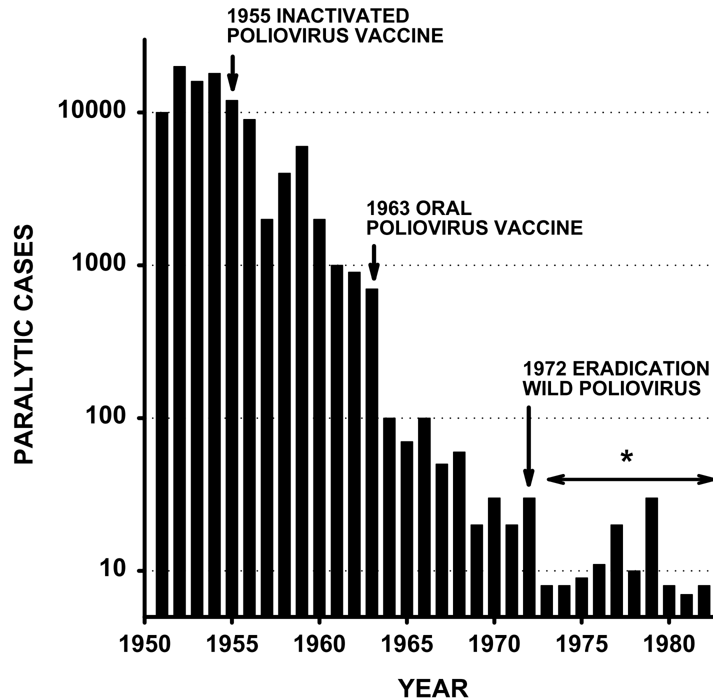

Fig. 4. Annual reported cases of paralytic poliomyelitis in the United States, 1951–1982. For the years 1973–1982, marked with an asterisk, residual cases are either vaccine-associated or imported, with the exception of a 1979 outbreak in the unvaccinated Amish population resulting from an importation. After [6]

poliovirus immunization surveys to determine the percent of children receiving OPV, and serosurveys of immunityindicated that there was a residual susceptible population estimated at up to 10,000,000 [5, 12, 15]. It was widely assumed that this pool of susceptible hosts would continue to circulate wildtype polioviruses indefinitely, and eradication was not contemplated. Under these circumstances, how could eradication occur?

Again, I would postulate that seasonality played a critical role in eradication [15, 16]. Figure 5 shows that, as for measles, poliovirus infections were highly seasonal, particularly in the northern United States. In Table 4, the seasonal curves are used to estimate the incidence of poliovirus infection in a hypothetical metropolitan area with a population of 10,000,000, both for the northern and the southern United States. Vaccine-induced reduction of susceptible individuals in such a population can be guesstimated to reduce the number of new infections per trough generation period below the threshold for virus perpetuation. When poliomyelitis incidence data for the period 1960 through 1972 are plotted by state (Fig. 6), it can be seen that each year a decreasing number of States reported paralytic polio. It can be surmised that, in area after area, the virus disappeared during the wintertime trough and was not introduced in the following summer, eventually leading to eradication. Although space does not permit, it is noted

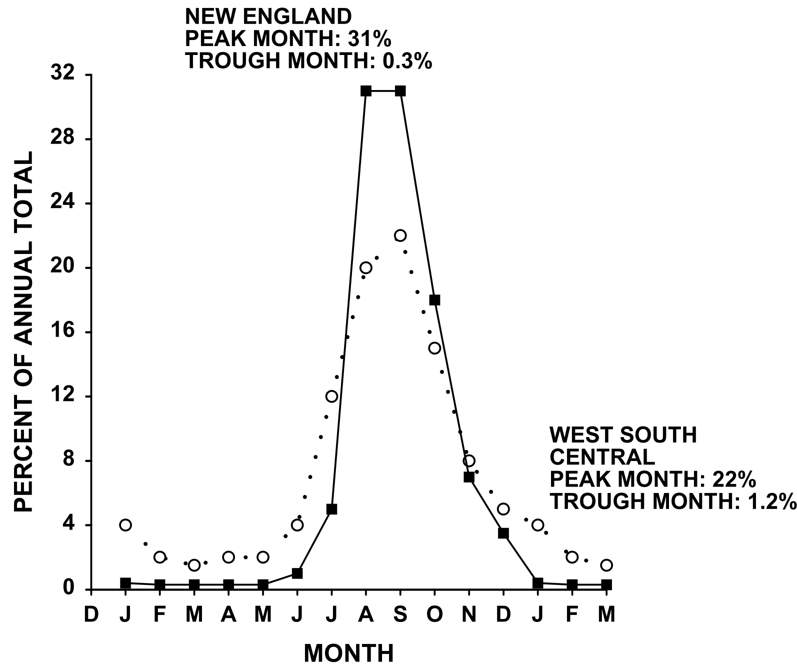

Fig. 5. Seasonal distribution of poliomyelitis (paralytic and nonparalytic) for two regions (New England and West South Central) of the United States, 1942–1951. After [23]

Table 4. Calculated number of poliovirus infections per generation period during the seasonal trough, in a population of 10,000,000 in the United States, prior to poliovirus vaccine and after the introduction of poliovirus vaccine (after [15])[a]

Parameter	Pre vaccine era 1950–1955	Post vaccine era 1960–1970
Total population	10,000,000	10,000,000
Susceptible population	2,200,000	360,000
Annual poliovirus infections	200,000	400
Infections per month at seasonal low (0.1%–0.4% of annual total)	200–800	0.4–1.6
Infections per generation period at seasonal low (10 day generation period)	70–280	0.1–0.5

[a]Susceptible population estimates based on the age distribution of poliomyelitis and upon serosurveys of poliovirus antibodies. Infections back-calculated from cases of paralytic poliomyelitis. Seasonal trough based on monthly distribution of poliomyelitis cases. Generation period based on studies of secondary polio cases in families. See [15] for references

that a similar phenomenon occurred with measles, but measles – with a greater transmissibility than poliovirus – was reintroduced after each fade out [15].

The elimination of wild poliovirus in the United States gave credibility to the extension of eradication. Major efforts were initiated in Central and South

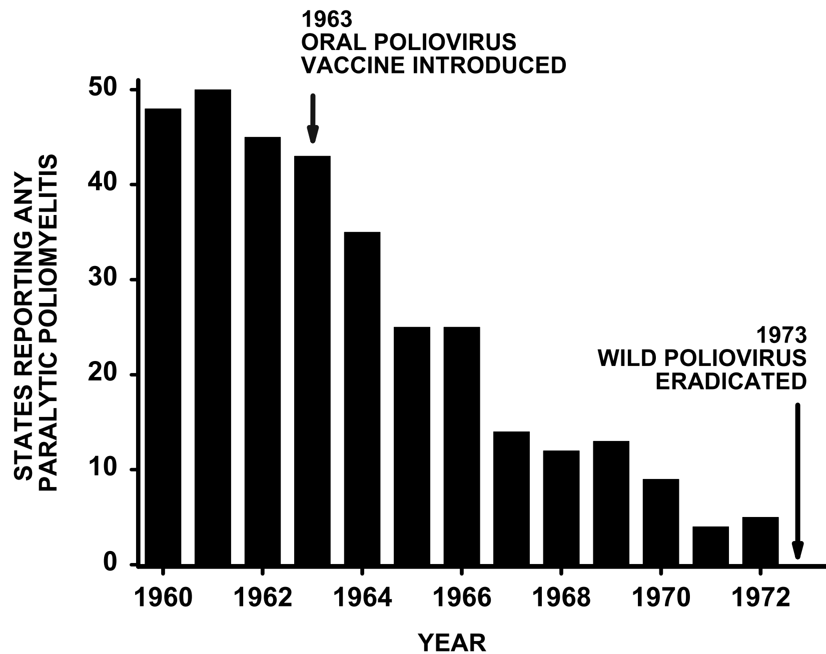

Fig. 6. The number of states reporting any cases of paralytic poliomyelitis, United States, 1960–1973, excluding imported and vaccine-associated cases. Based on data in [4]

America, leading to successful eradication in the 1980s. Emboldened by these successes, WHO embarked on global eradication, a goal that appears within reach within the next several years. The principal residual sites where wild poliovirus continues to circulate are Pakistan, India, and Nigeria, and it is likely that the absence of seasonality [7, 28] in these semi-tropical nations has been one of the impediments to eradication.

HIV and AIDS

One of the salient questions regarding the biology of HIV is: how did it emerge as a human virus? I will argue that the ability of HIV to cause persistent infections likely played a key role in its emergence, and is therefore worth a brief consideration in this essay on viral perpetuation.

Although circumstantial, the evidence is quite persuasive that HIV arose when a simian lentivirus, SIVcpz, jumped from chimpanzees to humans [9, 11, 13]. Many animal viruses cause zoonotic infections of humans but very few of them are subsequently transmitted from person to person. Most of those zoonotic viruses that are capable of limited human-to-human transmission exhibit marginal transmissibility, as evidenced by their containment using rudimentary quarantine measures and their fade out after a limited number of cycles. Examples are Crimean Congo hemorrhagic fever virus [20]; arenaviruses [14]; Ebola virus [22]; swine influenza virus in 1976 [17, 24]; and monkeypox virus [8]. The SARS coronavirus may be another example although to date it has not established itself as a human

Table 5. Speculative reconstruction of events following the hypothetical transmission of SIVcpz to humans[a]

Dates	Events
1915–1941	Transmission of SIVcpz to humans
∼1930–1980	HIV-1 maintained in rural villages in Africa
	HIV and AIDS are not recognized
1980–1985	AIDS recognized
	HIV-1 isolated
1980–2004	HIV-1 spreads rapidly through some urban and rural populations in Africa
	Global spread of HIV-1 and AIDS

[a]This reconstruction is based on data in [9, 11, 13, 18]

virus, even though it underwent at least 30 human-to-human passages in China in 2003 before being controlled by quarantine measures [27].

Rare indeed are those zoonotic viruses that become established permanently as human viruses. The best documented examples are influenza viruses, since avian influenza virus has on several occasions established itself in humans. It is noteworthy that, in several of these instances (such as the Asian pandemic of 1957 and the Hong Kong pandemic of 1968) the avian virus re-assorted with a human influenza virus, to produce a genetic chimera that endowed it with novel antigenic determinants, while maintaining the capability to transmit to humans [29].

These observations raise the questions as to how SIVcpz became established as a human virus. Recent studies have produced a speculative reconstruction of historical events following the hypothetical transmission of SIVcpz to humans (Table 5). Particularly relevant to this discussion is the inference that, following transmission to humans, SIVcpz was perpetuated as an unrecognized infrequent infection in rural villages in central Africa during the period 1930 to 1980 [18]. Different regions of the viral genomes of SIVcpz and of HIV-1 differ by 10%–25% [10] and it may be assumed that many of these changes were introduced during that 50-year interval. I speculate that some of these genetic changes have led to the metamorphosis of SIVcpz into HIV-1, to become an agent that can spread among humans with sufficient ease to be considered a virus of humans. If this is correct, then it would seem likely that the ability of SIVcpz to persist lifelong in the humans that it first infected might have provided an essential window of opportunity for a virus of chimpanzees to evolve into a human virus. Although tentative, these speculations offer interesting hypotheses for future research.

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The virus-immunity ecosystem

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Summary. The ecology of pathogenic viruses can be considered both in the context of survival in the macro-environments of nature, the theme pursued generally by epidemiologists, and in the micro-environments of the infected host. The long-lived, complex, higher vertebrates have evolved specialized, adaptive immune systems designed to minimise the consequences of such parasitism. Through evolutionary time, the differential selective pressures exerted variously by the need for virus and host survival have shaped both the "one-host" viruses and vertebrate immunity. With the development of vaccines to protect us from many of our most familiar parasites, the most dangerous pathogens threatening us now tend to be those "emerging", or adventitious, infectious agents that sporadically enter human populations from avian or other wild-life reservoirs. Such incursions must, of course, have been happening through the millenia, and are likely to have led to the extraordinary diversity of recognition molecules, the breadth in effector functions, and the persistent memory that distinguishes the vertebrate, adaptive immune system from the innate response mechanisms that operate more widely through animal biology. Both are important to contemporary humans and, particularly in the period immediately following infection, we still rely heavily on an immediate response capacity, elements of which are shared with much simpler, and more primitive organisms. Perhaps we will now move forward to develop useful therapies that exploit, or mimic, such responses. At this stage, however, most of our hopes for minimizing the threat posed by viruses still focus on the manipulation of the more precisely targeted, adaptive immune system.

Introduction

Vertebrates are large, complex multi-cellular, multi-organ systems. In nature, each of us functions as sets of ecological niches for the support of simpler life forms. Our most intimate passengers are the viruses, which only replicate in living cells. Many commensal organisms live in balance on skin and mucosal surfaces. Some

of these apparently innocuous companions are clearly held in check by specific host response mechanisms, as they will invade and cause disease and death when the capacity to mount an effective T cell response is compromised by, for example, HIV/AIDS . This is also true for highly adapted pathogens like Epstein Barr virus (EBV), which may cause infectious mononucleosis following initial exposure, then persists as a substantially latent infection that can drive oncogenesis subsequent to loss of immune control [83]. Clearly, EBV has developed molecular strategies through evolutionary time that facilitate both spread and long-term carriage [69].

It is, in fact, reasonable to think that the current character of the human immune system has been partly shaped by the continuing relationship with EBV and the other herpesviruses that persist in our lymphoid cells and neurons. There can be little doubt that the evolution of adaptive immunity, which first appears with the jawed fishes about 350×10^6 years ago [93], has been driven by the need to deal with infection [34, 68]. The molecular mimics of, for instance, cytokine and chemokine receptors that are found in the large, complex, DNA viruses indicates that the reverse may also be true for many one-host pathogens [94, 116]. Such stable parasitism reflects reciprocal relationships developed through the long march of phylogeny.

Emergence and persistence in macro and micro environments

Pathogens that grow in a variety of hosts are less likely to have achieved a long-term interaction with us and can thus be very dangerous when encountered for the first time. Much of Bob Shope's research career [109, 110] focused on arboviruses that are maintained in wildlife reservoirs and cause only incidental infection of humans. Many totally new viruses were discovered as a consequence of, for example, the Rockefeller Foundation-funded programs of 1950–1970. Some, like Ross River virus [41] were only found to be causative agents of human disease by retrospective analysis of stored serum samples. The arbovirology community of this era included many who were as at home in tropical rain forests as in the laboratory.

Unlike the human herpesviruses, the mosquito-borne viruses that are normally maintained in (for example) birds have been under no selective pressure to accommodate to human immune response mechanisms. The same may be true for many of the "emerging" pathogens that impact on humans and domestic animals suddenly, or sporadically, as a consequence of changes in culture, behaviour and/or environment. The need to deal effectively with this enormous spectrum of novel infectious agents is likely to have been one factor driving the extreme diversity of both the B cell (immunoglobulin) and T cell receptor (TCR) families [1, 7, 42]. Another may be the rapid variation associated with the error-prone copying mechanisms of some RNA viruses. Furthermore, as the T cells focus on complexes (epitopes) formed by the binding of processed viral peptides to major histocompatibility complex (MHC) molecules [131], the extreme polymorphism of the MHC [34, 92] can also be considered to reflect the evolutionary need to attach previously un-encountered spectra of peptides to one or another MHC glycoprotein.

The characteristics of viral emergence and persistence can thus be considered in two independent, though not necessarily unrelated, contexts. The first is in the broad environmental sense that considers such factors as climate, rainfall, forest management, vector and reservoir distribution, changing demographic profiles and so forth. The second concerns the environment within, whether "within" be infected cells and organs in arthropods, vertebrates or humans. Some determining factors are virus growth characteristics, virus escape mechanisms, immune receptor (antibody or TCR) specificity and diversity, immune selective pressure, antigen presentation characteristics, lymphocyte proliferation rates and the quality of immune effector function, both at the cell and the molecular level. When it comes to the practical consideration of developing protective vaccines, it is also necessary to think about the nature and durability of immune memory [21, 40, 115].

This brief discussion concentrates on adaptive immunity, the spectrum of precisely targeted host response mechanisms that maintain the functional integrity of the environment "within" subsequent to virus challenge. It ignores the innate immune system, which may be of great importance in the early stage of infection with some viruses, particularly the herpesviruses [71], but is neither conventionally antigen specific nor capable of generating the long-term memory that is the basis of immunization. Of course, both aspects of immunity work together. The themes of cytotoxic effector function [30] and localized cytokine production [95, 103], particularly γ-interferon (IFN-γ) and tumor necrosis factor-α (TNF-α), are shared by the cells of the innate and adaptive systems.

Plasma cells,B lymphocytes and antibodies

Antibody has been the traditional focus of virologists interested in the immediately practical concern of making effective vaccines. Techniques for generating strong serum antibody response to pathogens, or their products, were known through much of the 20th century, and provided our first opportunity to exploit immunotherapy with products like antitoxins and antivenenes. Many of the first immunologists were, in fact, called serologists. Early pioneering work on the immune system, including the discovery of the role of the plasma cell in antibody production [44] by Astrid Fagraeus (1913–1993), was done, for example, at the State Serum Institute in Copenhagen.

Measurement of serum neutralizing antibody is still the best correlate of vaccine-induced immunity for many viruses. In the main, the protective, virus-specific immunoglobulin (Ig) molecules are targeted to tertiary, conformed determinants of glycoproteins expressed on the surface of the virion [76]. Such pre-existing Ig may not completely block infection at (for example) mucosal surfaces [99], but it does prevent the systemic spread of blood-born virus to distal sites of potential pathology such as the large motor neurons in poliomyelitis. One of the challenges for immunologists is to develop strategies for maintaining high levels of mucosal antibody [72]. Can we hope to vaccinate against HIV if the virus cannot be stopped at the initial site of entry?

Antibody-mediated selection pressure drives the diversification of the influenza A viruses manifested as antigenic drift in the broader ecological context [46, 127], while the continuing emergence of antibody escape variants within an infected individual is a depressingly familiar characteristic [54, 63] of pathogens like HIV and hepatitis C virus (HCV). Recent strategies for developing neutralizing antibody response to (for example) the M2 channel protein that is expressed on the surface of the influenza A viruses [84] suggest that it may be possible to generate protective antibodies directed at conserved determinants expressed on molecular structures that have little, if any, capacity to vary. This would, of course, be the "holy grail" for HIV research [23].

Serum antibody is often detected indefinitely after vaccination or primary infection. Recent experiments have shown that B lymphocytes specific for vaccinia virus may be circulating in peripheral blood for as long as 50 years after exposure to the DryVax vaccine [31]. Vaccinia virus is not present in the normal human environment, and it is unlikely that (at least) most urban dwellers will have encountered even a distantly related poxvirus that infects, for example, domestic animals. Memory in the B cell/plasma cell compartment can apparently be maintained in the very long term without further challenge by the inducing antigen.

Antibody production is a property of plasma cells, the terminally-differentiated stage of the B cell lineage. During the acute phase of an infectious process, activated B cells/plasmablasts circulate in the blood and localize to various distal sites. In the viral encephalitides, for instance, B cells/plasmablasts can be seen to transit [32] from the blood to the central nervous system (CNS), where they become plasma cells and continue local antibody production in the long term [52, 98]. Persistent infection with a defective variant of measles virus in subacute sclerosing panencephalitis is characterized by massive, long-term local antibody production [114]. Subclinical infection of the CNS with an encephalitic virus can also lead to the sustained presence of neutralizing Ig in cerebrospinal fluid (CSF) at titers that are clearly discordant with levels in serum, providing a clear indication of local Ig synthesis in the brain [98].

Other B cells/plasmablasts find their home in the bone marrow (BM), a process that is clearly independent of antigen [60, 111] localization to that site. Long term Ig production seems, in fact, to be a function of the BM compartment [112]. The mammalian BM functions to provide continuous replacements for cells in the hemopoietic lineages, including naïve B cell precursors. Perhaps the spectrum of growth and differentiation factors that are required for this purpose also act to sustain the antibody-producing plasma cells [26].

Though we have been studying antibodies for a very long time, there are still big gaps in our understanding of topics like virus neutralization. The traditional neutralization assay done in tissue culture does not, for example, take account of the possible role of complement activation [132], or of opsonization and destruction by macrophages, mechanisms that are likely to be operational in the *in vivo* situation. The possible role of enhancing antibodies as a mechanism for promoting virus growth and damage in macrophages and epithelial cells has been

a major focus for those interested in hemorrhagic dengue [55]. Similar questions have been raised for HIV, though more in the context of promoting virus growth and persistence [80].The structural basis of antibody neutralization is clearly an important focus [8]. More research is being done on antibody neutralization, particularly as attempts are made to develop immunization strategies to limit the ravages of the AIDS pandemic [23, 70].

Helper and effector CD4$^+$ T cells

No long-term, protective antibody response is generated in the absence of CD4$^+$ helper T cell function. Viruses can promote some IgM production, but even the generation of substantial IgM titers depends on the involvement of helper T cells [104]. In general, the requirement is for "cognate help": the two categories of lymphocytes must interact directly via TCR-mediated CD4$^+$ T cell recognition of viral peptide in the binding site of the appropriate MHC class II glycoprotein on the surface of the B cell. Early IgA production may break this rule [105], but there is still an absolute requirement for the concurrent stimulation of CD4$^+$ T cells by other antigen presenting cells, particularly dendritic cells (DCs). The possible mechanism is that the T cells promote IgA production by B cells that have bound viral components via surface Ig and are in sufficient proximity to be stimulated by secreted lymphokines and cytokines. However, this is likely to be an exceptional situation.

Though a concurrent CD4$^+$ T cell response does not seem to be required for the development of an effective CD8$^+$ T cell response [14], it is clear that both the qualitative and quantitative character of virus-specific CD8$^+$ T cell memory may be compromised in the absence of concurrent CD4$^+$ T help [13]. This applies to both the generation and the recall of memory CD8$^+$ T cells. Unlike the B cell/antibody response, CD4$^+$ T help for the CD8$^+$ responders is thought to operate via the DCs, with the role of the CD4$^+$ T cells being to activate the DCs to be more effective antigen presenting cells [15, 100]. High-level virus persistence in the absence of CD4$^+$ T help is also associated with a progressive loss of functional capacity by CD8$^+$ T cell effectors [79, 82, 133]. This "immune exhaustion" effect is seen most clearly with LCMV, and is less apparent for persistent infections that are characterized by less fulminant antigen production [75, 117].

Activated CD4$^+$ T cells play a very important role as direct mediators of immune control in the host response to intracellular bacteria [67] and herpesviruses [81]. In general, a primary requirement for these CD4$^+$ T cell effectors [28] is the production of IFN-γ. Mice that are CD4$^+$ T cell deficient as a consequence of disruption of the H2I-A^b gene can only partially limit the lytic phase of murine γ herpesvirus 68 (γ HV68) infection, and succumb after about 100 days with a late-onset, wasting disease [25]. Experiments with the influenza A viruses suggest that CD4$^+$ T cells promote recovery by providing help for the antibody response [121], though there is other evidence that they can function directly in the site of pathology [136]. Selective priming of CD4$^+$ T cell memory can lead to a more rapid antibody response to Sendai virus, to greater localization of CD4$^+$ T cells to

the infected lung and to more rapid virus clearance [136]. Recent evidence with the mouse hepatitis coronavirus neurological disease model suggests that $CD4^+$ T cells can mediate virus clearance in the absence of antibody, but with substantially delayed kinetics (S. Perlman, personal communication).

Cytokine production by $CD4^+$ T cells can also have profound deleterious effects. Mice that lack $CD8^+$ T cells as a consequence of disruption of the β2-microgloubulin (β2-m) light chain of the MHC class I glycoprotein fail to clear lymphocytic choriomeningitis virus (LCMV) and develop a chronic, wasting disease [37]. This was shown to reflect the persistent stimulation of $CD4^+$ T cells by the otherwise non-pathogenic LCMV. Also, if mice acutely infected with LCMV (or with an influenza A virus) are dosed with a "superantigen" (staphylococcal enterotoxin B), the resultant, massive, cytokine "dump" by highly activated, virus-specific $CD4^+$ T cells can lead to death from TNFα-mediated shock [106, 134]. It is also possible that cross-reactive $CD4^+$ memory T cell stimulation [77] and the resultant cytokine release could be a factor in the hemorrhagic syndrome that can follow secondary infection with heterologous dengue viruses [87].

The great majority of autoimmune disease that are though to be T cell mediated have been associated with $CD4^+$ [16] rather than $CD8^+$ T cell response, though this perception may be changing [73]. Such syndromes may, of course, reflect the breaking of self-tolerance by exposure to molecular mimics of self-components expressed by invading viruses or bacteria [88, 108]. The broad alternative is that this apparent autoimmunity is directed at persistent, but as yet uncharacterised, viruses [114].

$CD8^+$ effector T cells

The $CD8^+$ T cell is the primary mediator of virus clearance in the acute phase of most infections, and can act in the absence of $CD4^+$ T help and antibody production to deal with at least some lytic viruses that lack a persistent phenotype [39, 126]. Though activated, virus-specific $CD8^+$ T cells are potent producers of cytokines [113], particularly IFN-γ and TNF-α, the principal effector mechanism in many infectious processes is thought to be cell-mediated cytotoxicity. Activated $CD8^+$ cytotoxic T lymphocytes (CTL) contain large intracytoplasmic granules that express the pore-forming protein, perforin, and a range of serine esterases, or granzymes [62, 74]. These discharge their contents at the "immunological synapse" that forms at the interface between the "killer" lymphocyte, and the infected cell, with the perforin and granzymes then acting synergistically to trigger the classical apoptosis pathway [45].

Apoptotic elimination can, if the perforin/granzyme pathway is disrupted, also be induced via the interaction of Fas ligand on the CTL with Fas expressed on the infected cell [120]. The latter mechanism may, however, be less precisely constrained by TCR/epitope recognition, and thus more likely to induce bystander killing of other cells that happened to have increased Fas expression [122]. Even in the absence of such "promiscuous" lysis, some immunopathology is an inevitable

consequence of any virus-specific CTL response [33, 90, 128]. The simultaneous elimination of large numbers of infected cells in (particularly) sensitive sites like the CNS can lead to massive functional impairment and even death.

The nature of the infectious process can determine the relative significance of different $CD8^+$ T cell effector mechanisms. While IFN-γ seems to play (at most) an ancillary role in the control of influenza pneumonia [50, 96], local production of IFN-γ by $CD8^+$ T cells is clearly important for the clearance of respiratory syncytial virus from the lung [90]. Another situation where IFN-γ produced by the $CD8^+$ T cell is the primary mediator of virus control is in the transgenic mouse, human hepatitis model studied by F. Chisari and colleagues [53]. Production of IFN-γ by $CD8^+$ T cells is also central to the limitation of alphavirus [18, 19] and enterovirus infections [101] in the central nervous system. Though inflammation may alter the normal profile [24, 97], neurons do not generally express MHC class I glycoproteins [124]. Any T cell-mediated control of neuronal infection is thus likely to work via locally secreted factors rather than by the precisely targeted, direct T cell/target contact that is required for cytotoxic elimination.

What we learned through the 1990's from experiments with genetically disrupted, "knockout" mice is that disabling molecular mechanisms that are thought to constitute the primary mode of virus control often serves simply to reveal the existence of potent, alternative, effector functions [36]. In the phylogenetic sense, it is easy to see the reason for this divergence. The large DNA viruses, such as the herpesviruses [49] and poxviruses [86], have evolved a number of strategies for defeating cell-mediated immunity. It is important, both for the survival of the host and the parasite in the evolutionary sense, that there should always be an alternative means of control, at least to the level that allows for some persistent virus production, or reactivation from latency.

T cell memory and the recall response

The development of FACS staining approaches utilizing tetrameric complexes [2] of MHC class I glycoprotein + peptide (tetramers) has greatly facilitated the analysis of both the effector and memory phases of virus-specific $CD8^+$ T cell responses [20, 40]. This technology has moved more slowly for the $CD4^+$ T cell subset [5, 6, 58], partly because the comparable MHC class II + peptide reagents are more difficult to produce [129], and partly because $CD4^+$ T cell responses can tend to be both more diverse and smaller in magnitude.

The most useful techniques for analysing $CD4^+$ T cell memory depend on the measurement of IFN-γ production by peptide-stimulated lymphocytes, measured either in a flow cytometric assay or by ELISpot analysis after 24–48 hours of *in vitro* culture [66]. Persistent $CD4^+$ T cell memory is, for example, found for adenoviruses in healthy humans [89]. Lack of progression to AIDS has been correlated with the continued presence of more HIV-specific IFN-γ than IL-10-producing $CD4^+$T cells in the peripheral circulation [91]. Priming $CD4^+$ T cell memory to a prominent Sendai virus epitope led to a more rapid antibody

response and enhanced virus clearance [136]. Low-level γHV68 persistence induced continuing $CD4^+$ and $CD8^+$ T cell responses that substantially prevented the establishment of further lytic, but not latent, infection following respiratory challenge of antibody-negative, μMT mice with the same virus [4]. Immune $CD4^+$ and $CD8^+$ T cells contributed to this protective effect in an additive way. Though adoptively-transferred $CD4^+$ and $CD8^+$ T cells promoted the recovery of μMT mice from influenza virus infection, the $CD8^+$ set was clearly more effective in this regard [51]. In general, we understand less about $CD4^+$ than $CD8^+$ T cell memory.

Persistent $CD8^+$ T cell memory can be demonstrated in both mice and humans following a single exposure to an inducing virus [38, 56, 61, 65, 78]. These long-lived T cells and their progeny express high levels of telomerase activity [57] though, under conditions of continuing antigen stimulation, telomere length may be shortened to the extent that clonal survival is impaired [102, 125]. The maintenance of $CD8^+$ T cell memory reflects the survival of clontotypes expanded during the initial, antigen driven phase of the host response [123], but does not seem to require either the persistence of the inducing epitope or even the continued presence of MHC class I glycoprotein [59, 85]. What does seem to be important is exposure to the cytokines IL-7 and IL-15, both during the acute response phase and in the long term. [17, 64, 107, 119, 135].

The recall of $CD8^+$ T cell memory can certainly provide a measure of protection against virus challenge [27], a possibility that is particularly attractive for viruses that vary their surface glycoproteins as a consequence of antibody-mediated selection pressure. Virus-specific $CD8^+$ T cell responses tend to be directed at peptides derived from conserved, internal proteins [12, 130], a situation that may be quite different from that found with $CD4^+$ T cell responses [22]. This cross-reactivity is, for instance, a good reason for thinking about the use of live influenza vaccines, combined with other mechanisms for boosting CTL memory [29, 43].

The problem with relying on the recall of $CD8^+$ T cell memory for protection is that, though the injection of peptide-pulsed cells is generally associated with rapid elimination [9], the recall of effective $CD8^+$ T cell memory to a distal site of virus growth is substantially delayed [35, 47]. When memory T cell numbers are at what might be thought of as physiological levels, there is a clear necessity for further proliferation in the lymphoid tissue, followed by emigration into the blood and localization to the target organ [48]. Even when memory T cell numbers are very high in, for example, the lung, a rapidly growing influenza A virus will still become fully established before $CD8^+$ T cell effectors operate to eliminate the infected cells and control the growth of the pathogen [29].

Thus, though vaccines directed at promoting $CD8^+$ T cell memory can limit the damage done by lytic viruses that do not have a capacity for persistence, they seem unable to prevent the establishment of persistent infections [4, 118]. This has been clearly demonstrated for monkeys primed with candidate HIV vaccines [3, 10]. The T cells function for a time to limit the extent of virus replication, but escape variants eventually emerge [11].

Conclusions

Some virus infections may be controlled by either an effective CD8$^+$ T cell response, or by a high quality B cell/antibody response that depends on CD4$^+$ T help. In general, however, the host response is optimally mediated by all three categories of immune lymphocyte operating together. This is clearly the case for the large DNA viruses, particularly the herpesviruses, which also require the involvement of cytokine-producing CD4$^+$ effector T cells [28]. Most of our successful vaccines to date depend on the capacity of a persistent neutralizing antibody response to limit systemic spread to distal sites of virus growth. Memory CD8$^+$ T cells may not prevent the establishment of an infectious process, though the more rapid recall of CTL effector function is likely to ameliorate the severity of pathology and consequent clinical impairment by speeding virus clearance [29]. Again, with viruses that have the capacity to persist, the available evidence suggests that an optimal vaccine will prime all the components of adaptive immunity [3].

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Host range, amplification and arboviral disease emergence

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Summary. Etiologic agents of arboviral diseases are primarily zoonotic pathogens that are maintained in nature in cycles involving arthropod transmission among a variety of susceptible reservoir hosts. In the simplest form of human exposure, spillover occurs from the enzootic cycle when humans enter zoonotic foci and/or enzootic amplification increases circulation near humans. Examples include Eastern (EEEV) and Western equine encephalitis viruses (WEEV), as well as West Nile (WNV), St. Louis encephalitis (SLEV) and Yellow fever viruses. Spillover can involve direct transmission to humans by primary enzootic vectors (e.g. WNV, SLEV and WEEV) and/or bridge vectors with more catholic feeding preferences that include humans (e.g. EEEV). Some viruses, such as Rift Valley fever, Japanese encephalitis and Venezuelan equine encephalitis viruses (VEEV) undergo secondary amplification involving replication in livestock animals, resulting in greater levels of spillover to humans in rural settings. In the case of VEEV, secondary amplification involves equines and requires adaptive mutations in enzootic strains that allow for efficient viremia production. Two of the most important human arboviral pathogens, Yellow fever and dengue viruses (DENV), have gone one step further and adopted humans as their amplification hosts, allowing for urban disease. The ancestral forms of DENV, sylvatic viruses transmitted among nonhuman primate reservoir hosts by arboreal mosquitoes, adapted to efficiently infect the urban mosquito vectors *Aedes aegypti* and *Ae. albopictus* during the past few thousand years as civilizations arose. Comparative studies of the sylvatic and urban forms of DENV may elucidate the evolution of arboviral virulence and the prospects for DENV eradication should effective vaccines be implemented.

Arthropod-borne viruses, or arboviruses, include many zoonotic pathogens that are transmitted among vertebrate reservoir hosts by mosquitoes, ticks or other biting arthropods [6]. Arboviruses also include important human pathogens that cause a variety of serious and sometimes fatal diseases, particularly in the tropics. To infect humans, these viruses use a variety of mechanisms ranging from simple spillover from enzootic cycles to adaptations that alter their host ranges to include domestic animals, and enhance their amplification in proximity to humans

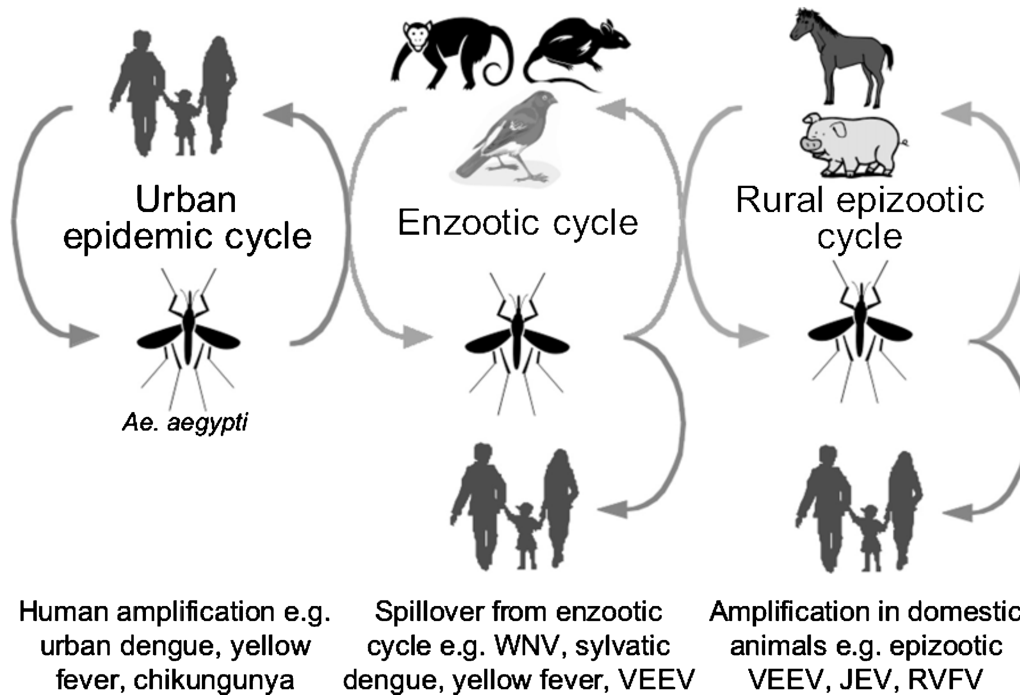

Fig. 1. Cartoon showing mechanisms of human infection by zoonotic arboviruses. At the center is an enzootic cycle, typically involving avian, rodent or nonhuman primates as amplification and/or reservoir hosts and mosquito vectors. Humans become infected via direct spillover when they enter enzootic habitats and/or when amplification results in high levels of circulation. Transmission to humans may involve the enzootic vector or bridge vectors with broader host preferences. At right, secondary amplification involving domestic animals can increase circulation around humans, increasing their chance of infection via spillover. In the case of VEEV, mutations that enhance equine viremia are needed for secondary equine amplification. At left, dengue and Yellow fever viruses can use humans directly for amplification, resulting in urban epidemic cycles and massive outbreaks. In the case of dengue viruses, humans also serve as reservoir hosts

(Fig. 1). In the most extreme example, dengue viruses (DENV-1–4, 4 different viruses) have evolved independently to adopt humans as their amplification and reservoir hosts, resulting in the emergence of tropical and subtropical pandemic disease during the past half century [10, 28]. This review describes examples of mechanisms of arboviral disease emergence and the effect of host range on human exposure and infection, with a focus on mosquito-borne viruses of the New World. Examples are largely drawn from the alphaviruses (*Togaviridae*: *Alphavirus*) [30] and flaviviruses (*Flaviviridae*: *Flavivirus*) [8], which, along with the bunyaviruses (*Bunyaviridae*) comprise the major taxa of arboviruses [6].

Direct arboviral spillover

Most mosquito-borne arboviruses utilize small mammalian or avian reservoir hosts (those responsible for long-term maintenance), which along with the vectors determine the distribution of transmission under normal circumstances (Fig. 1).

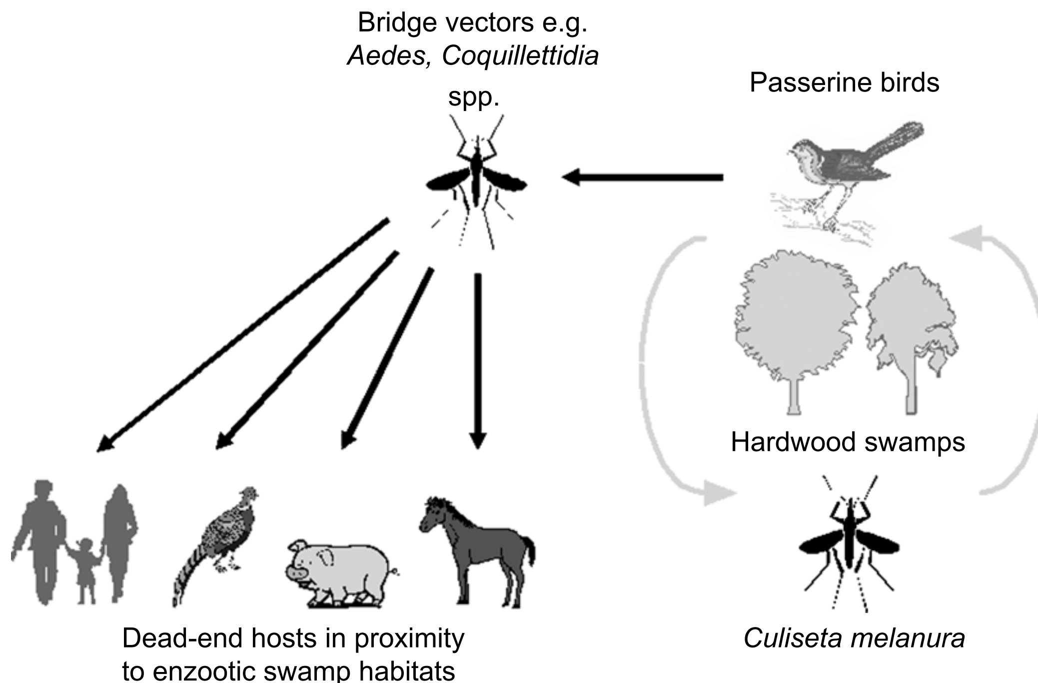

Fig. 2. Cartoon depicting transmission of Eastern equine encephalitis virus in North America. The enzootic cycle occurs in hardwood swamps where the ornithophilic mosquito *Culiseta melanura* transmits the virus among passerine birds that serve as reservoir and amplification hosts. Because *Cs. melanura* rarely bites mammals, other bridge vectors in the genera *Aedes*, *Ochlerotatus*, and *Coquillettidia* are believed to transmit this virus to humans, horses, pigs and gamebirds that suffer severe and often fatal disease. Because these hosts develop little or no viremia, they are considered dead-end and do not contribute to amplification. An exception is direct transmission via pecking among gamebirds, such as pheasants

Reservoir hosts also can serve as amplification hosts in the enzootic cycle and, along with the vector in some cases, maintain the arbovirus over time. In the United States, several of the most important human arboviral pathogens use avian reservoir hosts including Eastern (EEEV) and Western equine encephalitis viruses (WEEV) in the genus *Alphavirus*, and St. Louis encephalitis (SLEV) and West Nile viruses (WNV) in the genus *Flavivirus*. With the exception of EEEV, which uses *Culiseta melanura* as its principal vector in North America (Fig. 2), all these viruses use principal enzootic mosquito vectors in the genus *Culex*.

The vector habitat plays a major role in the distribution of these human diseases; equine and human WEE cases occur principally in agricultural ecosystems where *Culex tarsalis* breeds in irrigated settings and transmits the virus among birds [21]. Because this species exhibits a relatively wide host range, especially during the later part of the transmission season, direct spillover to humans and horses can occur after a mosquito becomes infected by a viremic avian blood meal, without the need for a bridge vector. Lagomorphs may also contribute to amplification and a secondary rabbit-*Aedes dorsalis* cycle has been documented; this species may also transmit the virus to humans. Because humans and equines

develop little viremia following infection with WEEV, secondary amplification is not thought to occur and cases only occur from direct spillover where amplification in avian populations has occurred. WEEV is genetically conserved in North and South America, with virus lineages spanning both continents. This implies efficient movement of viruses between the continents, presumably via infected birds [29].

In the case of WNV and SLEV in eastern North America, *Cx. pipiens* and *Cx. quinquefasciatus* appear to be the most important enzootic vectors [13, 25]. Other culicine species probably serve as secondary or bridge vectors, but *Cx. pipiens* and *Cx. quinquefasciatus* appear to account for most transmission among birds and to humans. These species are abundant in urban and suburban habitats where their larvae develop in sewers and other man-made aquatic habitats, resulting in the potential for large amounts of WNV spillover transmission to human urban residents. Unlike most arboviral diseases, drought conditions can promote SLEV and WNV amplification because *Cx. pipiens* and *Cx. quinquefasciatus* larvae prefer highly polluted water that occurs when water in storm sewers and other bodies stagnate. Humans and equines produce little viremia, so WNV cases are limited to locations where efficient amplification in avians occurs, followed by spillover to humans and other hosts. In the case of WNV in North America, severe disease and mortality also occurs in some avian amplification and reservoir hosts [12]; this may reflect a lack of selection for resistance among avian species exposed to WNV only since 1999, and possibly a lack of selection for WNV attenuation by declining host populations.

In contrast to WEEV, WNV and SLEV, the distribution of EEEV is restricted primarily to hardwood swamp habitats in coastal and inland locations of eastern North America (Fig. 2). Overwhelming evidence indicates that *Cs. melanura*, the principal enzootic vector, transmits EEEV among a variety of avian reservoir hosts [24, 31]. Unlike *Cx. tarsalis, Cx. pipiens* and *Cx. quinquefasciatus*, *Cs. melanura* has a very narrow host range and bites almost exclusively birds. This ornithophilic host preference is probably the single most important reason why there are usually few human EEE cases in North America, compared to WEEV, WNV and SLEV that have caused outbreaks involving up to thousands of cases per year. For transmission to equines, humans, pigs and other domestic animals, EEEV probably relies on bridge vectors such as *Coquillettidia perturbans*, *Aedes canadensis*, *Cx. salinarius*, *Ae. sollicitans*, and *Anopheles quadrimaculatus* that exhibit more catholic feeding behavior and are abundant during epizootics. Once infected, flocks of game birds such as pheasants and emus may undergo direct bird-to-bird transmission via pecking, resulting in high mortality rates. Factors limiting EEEV transmission to humans are the remote locations of many enzootic swamp foci and the inability of *Cs. melanura* to inhabit most urban and suburban habitats. However, as human populations continue to expand into more remote locations for recreation and housing, spillover of EEEV and human disease could increase.

Of particular interest with regard to EEEV is the potential for widespread outbreaks that could result if equines or other domestic animals could serve

as amplification hosts. The public health consequences of such a scenario are underscored by the history of massive outbreaks of Venezuelan equine encephalitis (VEE) in South, Central and North America, where equine amplification can result in up to hundreds-of-thousands of human cases via spillover from the epizootic cycle (see below) [33]. EEEV apparently has not adapted to produce equine viremia as high as that generated by some VEEV strains; understanding the genetic and ecologic bases of the ability of arboviruses such as EEEV and VEEV to adapt and undergo host range changes that lead to epidemic disease is essential to assessing the potential for future arboviral emergence.

An important flavivirus that causes human disease in the tropics via direct spillover is Yellow fever virus (YFV). The enzootic cycle in both Africa and South America involves nonhuman primates that serve as reservoir hosts and forest dwelling mosquito vectors such as *Aedes*, *Sabethes* and *Haemagogus* spp. [16, 17]. A secondary African savannah cycle involving similar kinds of vectors and nonhuman primates also has been described. In Africa, epizootic waves of disease in monkeys have been reported and many locations may be subject to temporary YFV extinction during interepizootic periods. Humans become infected in both Africa and South America when they contact the enzootic/epizootic habitats and are bitten by enzootic vectors. However, as described below, temporary amplification in humans via *Ae. aegypti* transmission also results in urban yellow fever.

Secondary amplification in domestic animals

Some arboviruses can achieve higher levels of circulation and infect more humans via secondary amplification in domestic animals. The phlebovirus (*Bunyaviridae*) Rift Valley fever virus is a good example; this virus is maintained primarily in sub-Saharan Africa by horizontal and transovarial transmission in mosquitoes, including *Aedes* and *Culex* spp. [2, 15]. Epizootics usually follow unusually heavy rainfall and involve infection of domestic ruminants including cattle, goats and sheep, resulting in high levels of horizontal transmission by mosquitoes to both domestic animals and humans, as well as direct human infections from the slaughter and handling of infected animals. This secondary amplification and transmission can result in epidemics in very arid locations not normally subject to arboviral outbreaks, such as Saudia Arabia [14].

Another example of secondary amplification resulting in human arboviral disease is Japanese encephalitis virus, a flavivirus that occurs in Asia and Oceania. Aquatic birds are the normal reservoir hosts and culicine mosquitoes that bite a viremic bird can infect humans, sometimes resulting in severe neurological disease, primarily in children [1, 5]. In many endemic locations, pigs are raised for food in close proximity to human habitations. When infected by a mosquito bite, pigs do not develop apparent disease but do become viremic, resulting in peridomestic amplification and increased risk for human infections (Fig. 1).

The above examples involve amplification of arbovirus transmission via domestic animals that are susceptible to most or all strains circulating. A more complex example is VEEV, a species including many serotypes that circulate

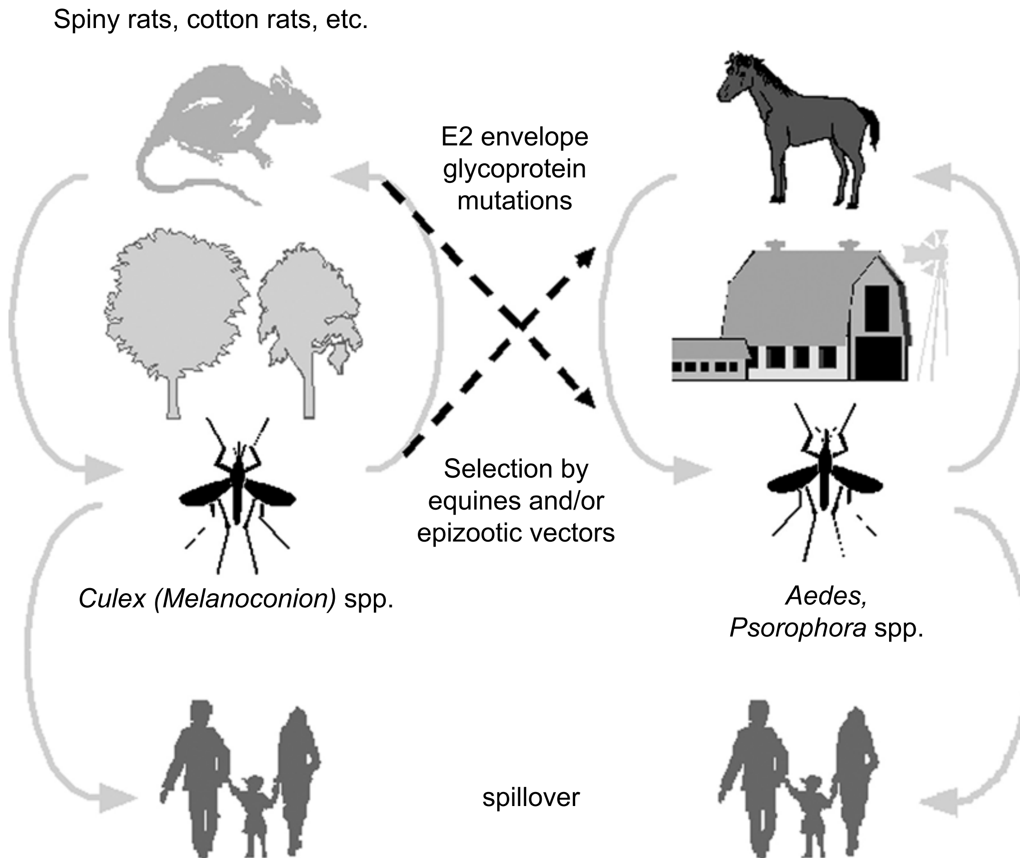

Fig. 3. Cartoon depicting transmission of Venezuelan equine encephalitis virus. The enzootic cycle at the left generally occurs in lowland tropical forest or swamp habitats where various members of the Spissipes section of the *Culex* (*Melanoconion*) subgenus transmit virus among rodent reservoir hosts. Human infection can occur via direct spillover when people enter enzootic foci and are bitten by enzootic vectors, which tend to have broad host ranges. Equine epizootics and epidemics involving up to hundreds-of-thousands of cases occur when enzootic ID strains mutate to generate the IAB or IC epizootic serotype and enhance their ability to produce equine viremia. This leads to intense transmission in agricultural settings, resulting in spillover to humans. Humans also develop high titered viremia and are therefore potentially capable of amplification, but epidemiological studies indicate no evidence of transmission in the absence of equines; this suggests that humans play a minor role as amplifiers

enzootically in much of Latin America, where they infect rodent reservoir hosts in stable forest or swamp transmission foci (Fig. 3) [34]. The mosquito vectors of these enzootic strains generally remain in the forest or swamp habitats. Therefore, human infection via direct spillover is usually limited to enzootic habitats, and large epidemics involving direct spillover have not been reported. However, certain epizootic strains of VEEV, comprising serotypes IAB and IC, produce massive epidemics and equine epizootics involving up to hundreds-of-thousands cases over a period of a few months to a few years. These strains achieve widespread and intense circulation by infecting equines and producing high titer viremia.

When large salt marsh or floodwater mosquito populations are present during rainy seasons, equines are extremely attractive hosts and an infected animal can generate hundreds of infectious mosquito vectors following blood feeding and extrinsic incubation, resulting in infection of additional equines as well as humans who live in agricultural settings (Fig. 3). However, epizootic transmission is unstable and is usually interrupted when most or all equines become infected and die or survive with immunity, and/or when mosquito populations decline following the onset of a dry season. Intervals of up to 19 years between outbreaks without evidence of serotype IAB or IC VEEV circulation in nature, led to investigations of the relationships between enzootic and epizootic strains. Evidence has accumulated supporting the hypothesis that one of many enzootic subtype ID lineages periodically mutates to produce the epizootic strains that initiate outbreaks [11, 20, 22, 23]. None of the enzootic variants is capable of amplifying in equines in their normal state [26, 32], so the generation of epizootics relies on small numbers of critical mutations in the E2 envelope glycoprotein of the enzootic progenitors that augment the ability of VEEV to produce equine viremia [6a]. Other evidence indicates that, in some cases, VEEV adapts to infect more efficiently the epizootic mosquito vectors that transmit during outbreaks. This adaptation is also mediated by mutations in the E2 glycoprotein that forms the spikes on the virion surface and probably interacts with specific cellular receptors of equines and mosquitoes [3, 4, 6a].

As mentioned above, it is unknown whether other arboviruses, such as EEEV, have the potential to adapt for amplification in domestic animals such as RVFV, JEV and VEEV. Understanding the genetic basis of arboviral host range and viremia phenotypes is critical to assessing the potential for future epidemics via secondary amplification in domestic animals.

Direct amplification of arboviruses by humans

Only a few arboviruses appear to use humans directly as amplification hosts, but this emergence mechanism can have dramatic implications for public health. One example is YFV, which has caused massive urban outbreaks in Africa and the Americas dating back at least several centuries [16, 17]. *Aedes aegypti* can transmit YFV among human amplification hosts, and its use of artificial water containers as breeding sites led to its efficient dissemination by sailing ships, probably beginning in the 16th century. Transovarial transmission of YFV in this vector may have also facilitated its transport.

Yellow fever virus is believed to have originated in Africa and to have been introduced into the Americas with the advent of regular transatlantic shipping hundreds of years ago [27]. Then, periodic YF epidemics in both temperate and tropical locations occurred in the Americas, particularly in port cities, until an *Ae. aegypti* eradication campaign eliminated this urban vector from most of Latin America during the mid-20th century. However, African outbreaks continued periodically during this era. There is no evidence of major strain variation or adaptive mutations involved in the initiation of the urban *Ae. aegypti*-human cycle, although

the strains involved in New World epidemics prior to the eradication campaign are not available for genetic analysis. Surprisingly, despite the reinfestation of most of Latin America by *Ae. aegypti* during the past 30 years, major urban epidemics have not recurred in the New World. A possible explanation is that only the African YFV strains are capable of initiating urban transmission via efficient infection of *Ae. aegypti*, with the American strains only producing spillover, endemic transmission in sylvatic habitats.

The most devastating examples of arboviruses adapting to urban transmission are dengue viruses (DENV), which produce most of the human arboviral disease. Four distinct DENV serotypes have been identified (DENV-1–4) and all can produce a highly debilitating febrile illness that can progress to hemorrhagic fever and fatal shock [7]. DENV circulates among human reservoir and amplification

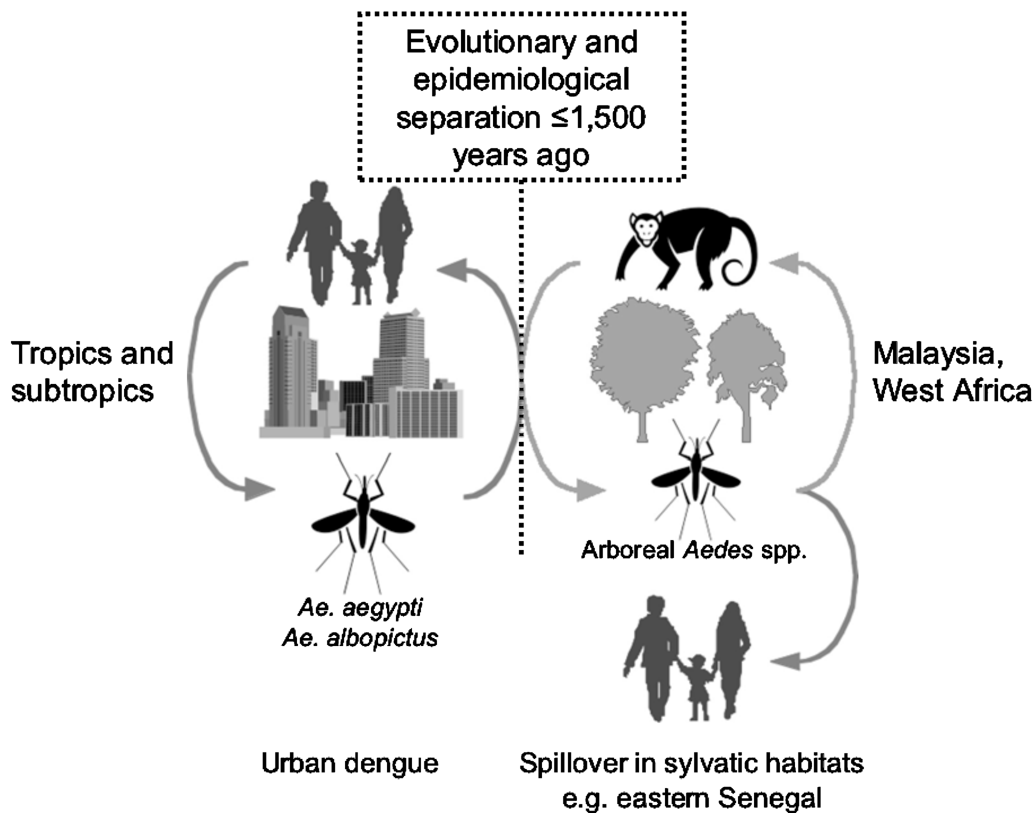

Fig. 4. Cartoon depicting dengue virus transmission cycles. Right, sylvatic, enzootic cycles of DENV-1, -2 and -4 in Malaysia, and DENV-2 in West Africa, involving nonhuman primates as reservoir hosts and arboreal *Aedes* spp. as vectors. These sylvatic cycles are believed to be ancestral, and human infection with sylvatic strains via spillover has been documented in Senegal [23a, 23b, 35]. The sylvatic cycles of all 4 serotypes in Malaysia or elsewhere in Asia are believed to have given rise independently to endemic/epidemic cycles involving humans as amplification and reservoir hosts, and the peridomestic *Ae. aegypti* as the principal vector. The sylvatic and endemic cycles then became independent, both evolutionarily and ecologically, during the past 1500 years

hosts in most tropical areas of the world, and *Ae. aegypti* is the principal vector, with *Ae. albopictus* and other subgenus Stegomyia species serving as secondary vectors (Fig. 4). Although several distinct lineages of DENV serotypes circulate in different parts of the world and have complex patterns of movement and introductions, all share this basic peridomestic transmission cycle. In contrast, genetically and ecologically distinct DENV strains have been isolated in West Africa and Malaysia, where they circulate among nonhuman primates and are transmitted by arboreal *Aedes* spp. mosquito vectors. These sylvatic strains are believed to be the ancestors of the endemic or epidemic strains that use human hosts, and each DENV serotype is believed to have emerged from the sylvatic cycle by changing its host range to humans, and its vector range to *Ae. aegypti*, during the past few thousand years [28]. To determine whether adaptation was required for these host range changes, the susceptibilities of *Ae. aegypti* and *Ae. albopictus* have been tested using both endemic and sylvatic DENV-2 strains. The consistently higher susceptibility of these vectors to the endemic strains suggests that DENV emergence relied on mutations that enhanced its ability to infect these peridomestic species [18]. Interestingly, the urban DENV vector subspecies *Ae. aegypti aegypti* is also believed to have originated in Africa from a tree hole-dwelling ancestor, *Ae. aegypti formosus*; the urban form adapted to breed in artificial containers and became endophilic, probably originally in Africa [19]. However, the sylvatic ancestral form, *Ae. aegypti formosus*, is not believed to be the principal vector of sylvatic DENV-2 in West Africa.

An important unanswered question regarding DENV evolution and emergence is the effect of its host range changes on virulence for humans. Sylvatic DENV-2 strains are known to infect humans in Senegal, with several reports of associated dengue fever-like illness [23a, 23b, 35]. However, it is unknown if human infections with sylvatic DENV strains produce viremia sufficient for transmission by periodomestic vectors, such that adaptation to humans would not be required to initiate a human cycle. Adaptation of DENV for enhanced human viremia, if required for peridomestic transmission, could be associated with more severe disease. Understanding the effect on human virulence of the viral host range changes from nonhuman primates to humans, and of *Ae. aegypti* from the arboreal treehole habitat to peridomestic settings, should elucidate trends in the evolution of arboviral virulence and facilitate predictions of the effect of future ecologic changes on human health.

Another important implication of DENV emergence from sylvatic cycles is related to vaccine development. If one or more of the vaccine candidates currently under development and clinical trials prove effective at preventing human infection, eradication of the endemic DENV cycles may be feasible because there is no non-human reservoir host. However, the possibility would remain that the sylvatic cycles could lead to re-emergence via DENV adapting again to *Ae. aegypti aegypti* and possibly to human hosts. Therefore, understanding both quantitatively and qualitatively the genetic determinants of these adaptations and the probability of their recurrence is critical to predicting the feasibility of human DEN eradication.

This example of urban DENV emergence via host range changes underscores the threat represented by RNA arboviruses due to their ability to rapidly mutate and exploit new ecologic opportunities [9]. As humans continue to invade and alter the habitats where arboviruses circulate, especially via deforestation and urbanization, selective pressures will mount for these viruses to adapt to peridomestic vectors, humans and/or their domestic animals as vectors, reservoir and amplification hosts, respectively. Both retrospective and experimental studies of arbovirus evolution, host range and adaptation are needed to improve our ability to predict disease emergence and to develop new intervention strategies.

Acknowledgements

This review is dedicated to the memory of Dr. Robert Shope, a wonderful friend, colleague and mentor to the author for over 20 years. Bob's pioneering studies of neotropical arboviruses in Brazil led to seminal discoveries on the ecology of enzootic VEE and EEE viruses, and also set the stage for advances in alphavirus systematics. These topics later became of intense interest to the author and the subject of much of his research. Bob also assembled the World Reference Center on Arboviruses, which contributed countless alphavirus and flavivirus strains used in the author's research on arbovirus evolution. His insightful thinking about the emergence of viral diseases strongly influenced the research directions of the author for the past 15 years. As a faculty member at UTMB since 1995, Bob served as an informal mentor to the author, providing invaluable advice that led to a smooth transition into a faculty role in academia. He was also a direct collaborator and leader in the development of alphavirus biodefense research initiatives that have transformed the research landscape at UTMB.

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Regulation of Rodent-Borne viruses in the natural host: implications for human disease

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Summary. Prevalence and transmission rates of rodent-borne viruses within host populations vary in time and space and among host-virus systems. Improving our understanding of the causes of these variations will lead to a better understanding of changes in disease risk to humans. The regulators of prevalence and transmission can be categorized into five major classes: (1) Environmental regulators such as weather and food supply affect transmission rates through their effect on reproductive success and population densities. (2) Anthropogenic factors, such as disturbance, may lead to ecosystem simplification and decreased diversity. These changes favor opportunistic species, which may serve as reservoirs for zoonotic viruses. (3) Genetic factors influence susceptibility of mice to infection or capacity for chronic shedding and may be related to population cycling. (4) Behavioral factors, such as fighting, increase risk of transmission of some viruses and result in different patterns of infection between male and female mice. Communal nesting may result in overwinter transmission in colder climates. (5) Physiologic factors control host response to infection and length of time the host remains infectious. Risk prediction is difficult because these regulators are numerous and often interact, and the relative importance of each varies according to the host species, season, year, and geographic location.

Introduction

In the collection of papers included in this special issue, we seek to further our understanding of the emergence and persistence of infectious viral diseases "from nature" that impact human health. Our common objective, to alleviate human suffering and improve public health, can be approached at multiple levels. The treatment of disease in humans, although important, should be a last resort. Elimination of the disease agent from nature usually is not possible. A third approach, which often is much more achievable, is to prevent the disease agent from making the jump from the natural reservoir to humans. This approach requires accurate risk prediction and development and targeting of effective preventive measures

which, in turn, require a detailed knowledge of the ecology and epizootiology of the hosts and vectors of zoonotic viruses. Studies of the dynamics of viral infection in natural host populations require a wide variety of methodologies, many of which are not commonly taught to students in public health science. Nevertheless, as human populations grow and continue to expand into formerly undisturbed natural habitats, these studies become increasingly important. Their successful accomplishment will require the collaboration of scientists from a variety of disciplines, including virology, immunology, ecology, zoology, geography, and mathematics. In this review, I will draw upon data from recent and on-going multidisciplinary studies that have increased our understanding of the regulation of rodent-borne viruses in natural host populations and relate these findings to the understanding and prevention of human disease.

Factors that influence or "regulate" the transmission and prevalence of infection of rodent-borne viruses in natural hosts are numerous and cover the full range of the biology and ecology of the host species. The space allotted to this article will allow only a general coverage of the major categories of these factors and a few examples of each.

For purposes of this article, I will define regulators as those factors that affect the transmission and prevalence of rodent-borne viruses in their natural hosts. In this context, regulation is not precise in the way that a precision instrument regulates temperature, pressure, or flow rate. Regulators in natural systems are factors that influence outcomes in ways that are difficult to quantify; their total effects vary according to their interactions with a myriad of other ecological regulators, making outcomes difficult to predict. Nevertheless, the better we understand these "regulators" the better we will be able to predict times and places of increased risk for zoonotic diseases or to develop effective interventions and target them appropriately. For convenience, I have divided factors that contribute to the prevalence and transmission of rodent-borne viruses in their hosts into five general, but overlapping, classes (Table 1). I will briefly describe each of these classes and provide illustrations, drawing from experience with the hantaviruses and arenaviruses.

Table 1. Principal categories of regulators of viral transmission in natural host populations

I.	*Natural environmental*:
	Weather, habitat quality, food supply
II.	*Anthropogenic*:
	Human disturbance or alteration of habitat
III.	*Genetic*:
	Variation among individuals or populations
IV.	*Behavioral*:
	Actions that affect transmission
V.	*Physiological*:
	Physiological predisposition or response of organism to infection

Environmental regulators

Environmental regulators such as weather, habitat quality, and food supply affect transmission rates through their effect on reproductive success and population densities. Hantavirus pulmonary syndrome (HPS) is a severe respiratory disease with high mortality [15, 25]. The great majority of HPS cases in the United States are caused by Sin Nombre virus (SNV), which is hosted by the deer mouse (*Peromyscus maniculatus*). It is widely believed that the first recognized outbreak of HPS in the southwestern United States in 1993 was associated with unusual environmental conditions that resulted in high risk of human disease [41]. The often repeated story is that the 1993 El Niño Southern Oscillation (ENSO) event brought unusually high rainfall to the arid Southwest, populations of deer mice increased dramatically, prevalence of infection increased as a consequence of these high population densities, the probability of humans encountering infected mice increased, and this produced the outbreak of HPS [41]. This scenario is logical but has been largely conjecture, because no one was specifically measuring deer mouse populations at the time. Since then, however, investigators supported by the Centers for Disease Control and Prevention have been tracking deer mouse population density and prevalence of infection with hantaviruses at several collection sites in the western United States [14, 32]. Figure 1A–C show the relationship between numbers of HPS cases in the southwestern United States during 1994–2003 and deer mouse populations, prevalence of infection with SNV using, as an index, values from four mark-recapture plots in northwestern New Mexico. There was another ENSO event in 1997, and investigators recorded increased rainfall, increased cover of green vegetation, and increased numbers of deer mice preceding increased numbers of HPS cases in 1998 Fig. 1A [41]. As predicted, the increased numbers of deer mice was followed by an increase in the prevalence of infection in host populations (with a delay of about a year; Fig. 1B). Nevertheless, neither the increase in deer mouse density nor the increase in prevalence above background levels temporally coincided with the increase in human cases. Perhaps most important is the comparison with the absolute number of infected deer mice (Fig. 1C). This index remained low when the number of human cases was low, but once the index rose above a certain threshold, human cases simultaneously increased. Although there does not appear to be a linear relationship (at this index site), this index remained above threshold levels throughout the period of increased risk in the Southwest. These observations allowed the successful prediction of high disease risk for human populations in the spring of 1998 [8, 9] and the spring of 1999 [9].

Thus it appears that rainfall was an environmental regulator of SNV transmission in rodent populations, and although the quantitative relationship was obscure, in qualitative terms, more rain appeared to translate to more virus and higher risk. Nevertheless, generalizing that conclusion even to other areas of the Southwest might not be appropriate.

At a grassland site in southeastern Colorado, investigators have been monitoring rodent populations and SNV since 1994 [3, 4]. Pinyon Canyon Maneuver Site

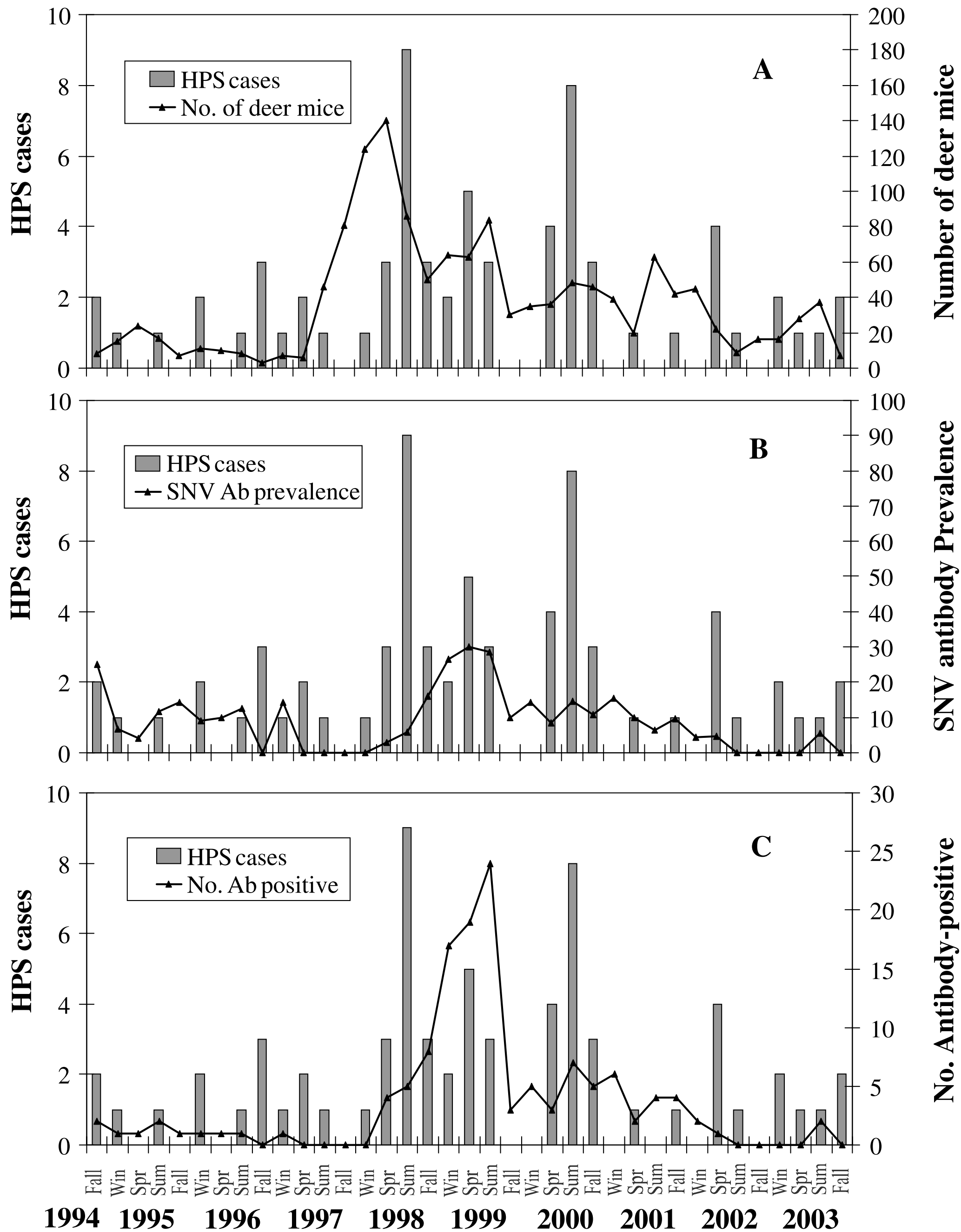

Fig. 1. Quarterly numbers of cases of hantavirus pulmonary syndrome (bars) compared with statistics describing host (deer mouse) populations at two long-term, mark-recapture sites in northwestern New Mexico, 1994–2003: (**A**) absolute numbers of deer mice captured, (**B**) prevalence of antibody to Sin Nombre virus in deer mouse populations, (**C**) absolute numbers of antibody-positive deer mice captured. After Yates et al. [41]

(PCMS) is a scrub grassland site with a different climatic regime from northwestern New Mexico. Spring and summer at PCMS have relatively high rainfall (50-yr means = 108 and 115 mm, respectively), while fall and winter are dry (means = 39 and 34 mm, respectively). Temperature patterns show a warm spring and summer (mean maximums = 27 and 32 °C; mean minimums = 7 and 13 °C) and a cold fall and winter (mean maximum = 15 and 12 °C; mean minimum = −4 and −7 °C). Thus the autumn and winter are cold and dry while spring and summer are warm and rainy. Deer mouse population dynamics at PCMS showed strong seasonal variation (Fig. 2A). There was a nadir in summer; populations increased in fall to a winter peak as the young of the year entered the trappable population, then declined again in spring to a summer trough. There were two exceptions to this pattern. In the fall of 1997 when the population should have begun to increase, it abruptly crashed. This crash coincided with a cold autumn, when rainfall was >300% of the normal value. The population recovered to resume its normal cycle

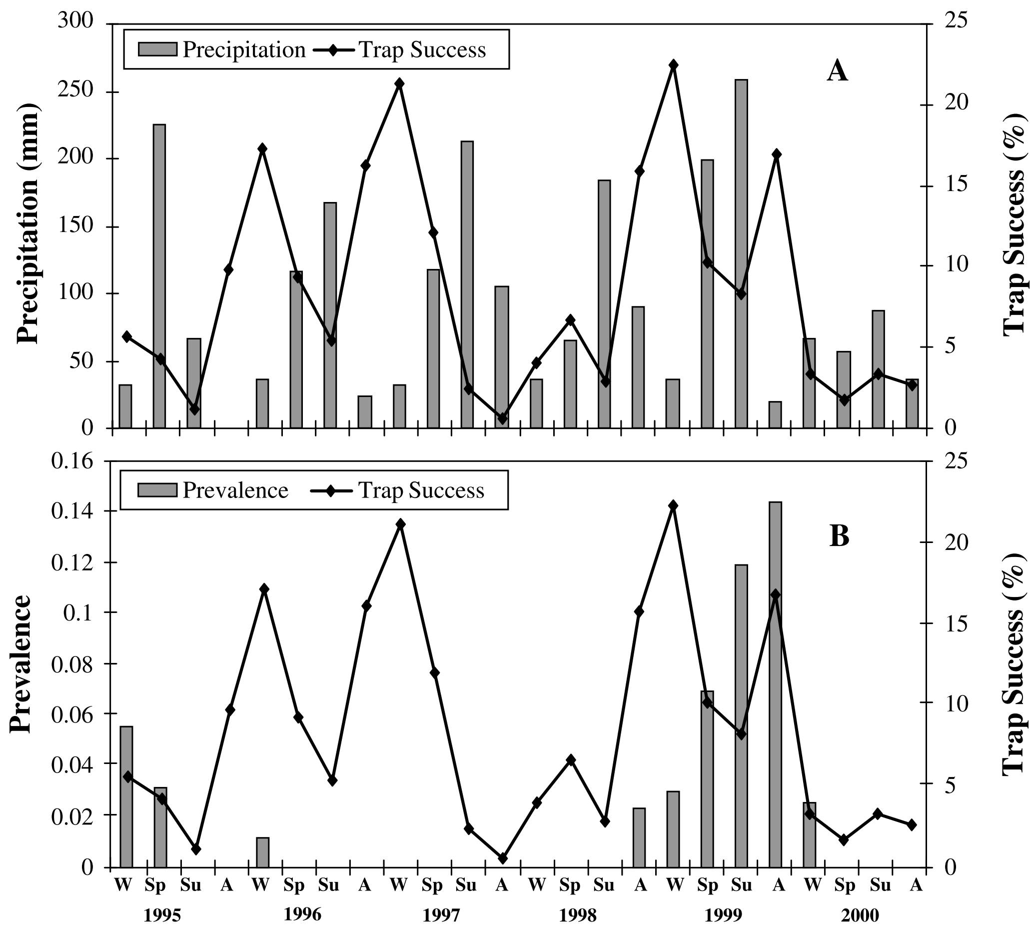

Fig. 2. Population dynamics of deer mice compared with (**A**) quarterly cumulative precipitation and (**B**) prevalence of antibody to Sin Nombre virus at a mark recapture site in southeastern Colorado. After Calisher et al. [3, 4]

in 1998 and into 1999, but the population abruptly crashed again in the winter of 2000, coincident with rainfall that was 150% of normal. The population showed no sign of recovery the following fall, following an extreme drought in spring and summer when rainfall was only 40% of normal. Thus, in the summer, *low* rainfall was associated with a negative effect on deer mice and, in the winter, *high* rainfall had a negative effect.

These host population dynamics appeared to have a great effect on the prevalence of SNV (Fig. 2B). Virus that was present at moderate prevalence disappeared from the population coincident with the drought and population nadir in summer 1995, was still absent during the population crash of 1997, but had recovered by 1999. The virus again appeared to become locally extinct following the population crash in winter 2000.

An important lesson from these observations is that regulators cannot be viewed independently. The effect of one important environmental regulator (rainfall) may vary, and even reverse direction, depending upon the season and upon its interaction with other environmental factors, such as temperature.

Anthropogenic regulators

Anthropogenic disturbance can result in dramatic changes in environmental conditions to which populations must adapt, move on, or die out. Certain opportunistic, more generalist, species may thrive under such disturbed conditions while more specialist, sylvatic species cannot survive. Thus the composition of rodent assemblages changes, usually becoming relatively species depauperate, restricted to a few opportunistic species whose population densities may increase dramatically under release from competitive pressures. This has been shown repeatedly, in relation to agriculture, ranching, and deforestation [7, 12, 27, 36, 37]. How might such disturbance affect viral infection in rodent hosts and subsequent risk to humans?

Kuenzi et al. [26] have been studying deer mouse population dynamics in sylvan and peridomestic habitats since 1996. They found several differences in populations inhabiting disturbed peridomestic situations as compared to those in more natural sylvan sites (Table 2). For example, the breeding season was about 2 months longer in peridomestic sites, and the prevalence of infection with SNV was 50% greater in deer mouse populations in peridomestic sites. Most

Table 2. Characteristics of deer mouse populations: Montana, 1996–1999, after Kuenzi et al. [26]

	Peridomestic	Sylvan
Mean breeding season (months)	~8.5	~6.5
Mean antibody prevalence	24.5% (490/2003)	16.5%* (302/1845)

*$p < 0.0001$ Chi square with Yates correction

human exposures to SNV occur in the peridomestic environment (Centers for Disease Control and Prevention, unpublished data). Thus anthropogenic disturbance can be a regulator of hantavirus infection in hosts, and subsequent risk to humans.

Decreasing natural biodiversity (usually brought about through anthropogenic disturbance) has been hypothesized to result in increasing risk for various diseases in human populations. This concept has been frequently cited in the popular press, and there has been some treatment of the hypothesis in the scientific literature [19]. Nevertheless, such treatments have remained largely theoretical, because few data are available for testing hypotheses. Using Lyme disease as a model system, Ostfeld and others [33, 34, 38] developed a general theoretical model for vector-borne diseases (the dilution effect hypothesis). The basic theory is that vertebrate communities with high species diversity will contain a greater proportion of incompetent reservoir hosts that deflect feeding vectors away from the most competent reservoirs, thereby reducing both infection prevalence in the vector and human disease risk [33].

Hantaviruses are frequently transmitted by aggressive encounters between individual rodents [18, 31]. Because most rodent species are dead-end hosts for hantaviruses, we hypothesized that hantaviruses might be transmitted more efficiently in rodent communities of lower diversity. We tested this hypothesis by relating the average antibody prevalence in the dominant hantavirus reservoir species to the average diversity indices at our longitudinal study sites in the southwestern United States. The Simpson's diversity index calculated for the rodent assemblage at each site explained 86% of the variation in hantavirus antibody prevalence (Fig. 3). This analysis provides evidence that simplification of ecosystems may result in increased transmission of some directly transmitted rodent-borne viruses.

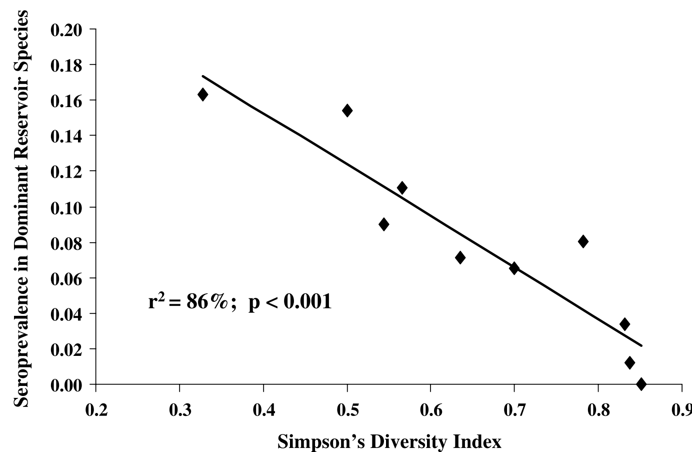

Fig. 3. Prevalence of antibody reactive with Sin Nombre virus in the principal host species compared with diversity of the small mammal assemblage at 10 mark-recapture sites in the southwestern United States, 1994–2000

Genetic regulators

The susceptibility and response of a reservoir host to infection with a zoonotic agent is largely determined by genetics [10]. Perhaps the most fundamental example of this is the coevolved pattern of asymptomatic, chronic infection and long-term viral shedding characteristic of hantavirus-host systems. This close co-adaptation is highly specific, in some cases even to the level of subspecies. The cotton rat (*Sigmodon hispidus*) has been divided into 12 geographically restricted subspecies in North America [20]. Black Creek Canal hantavirus is found only in association with *S. h. spadycipygus*, which is restricted to extreme southern Florida. Muleshoe hantavirus appears to be associated with *S. h. texianus* which occurs in eastern and central Texas, Oklahoma, and Kansas. Laguna Negra hantavirus is found only in association with populations of *Calomys laucha* in Paraguay, Bolivia, and N. Argentina [21, 35, 40]. A disjunct population of the same species in central Argentina appears to be free of infection with Laguna Negra virus (J. Mills, S. Levis, unpublished data). The experiments have not been done to determine whether this situation is a historical accident or due to genetic differences in susceptibility among populations of *C. laucha* [28]. In one case however, genetic differences within the same geographic population of a host seem to determine response to infection with an arenavirus. Based on results of laboratory inoculations, Johnson [22] proposed a model of Machupo virus infection in its host, *Calomys* sp. Laboratory studies indicated that Machupo virus was transmitted venereally among adult mice; and that newly infected dams gave birth to chronically infected pups. For adults however, there was a genetically controlled, split response to infection. Type B individuals cleared infection and had normal litters; type A's became chronically infected and females were effectively sterile. The varying interactions of the virus with these two genotypes were hypothesized to result in a cycling of host populations and to consequently varying risk to humans.

Mammalogists often classify small mammals into two categories based on life-history patterns [17]. Mammals representing generalist, opportunistic species are relatively common, highly fecund, rapidly maturing, highly mobile, and they are habitat and dietary generalists. These species often take advantage of disturbed conditions, reproducing to very high densities in a short period of time. These species are generally favored by disturbed, low-diversity conditions which they quickly colonize and in which they may reach high densities while conditions are favorable. On the other hand, specialist species are less fecund and relatively slow growing, making a larger investment in parental care. They usually require a specific and relatively narrow range of stable environmental conditions and food resources, are found in conditions of high diversity, and usually disappear following anthropogenic disturbances. Opportunistic, anthrophilic species make up a relatively small proportion of the 1000+ recognized species of Murid rodents [39]. Yet, a large percentage of reservoir hosts for rodent-borne viruses are what would be considered opportunistic species. Of 32 recognized hantavirus and arenavirus hosts, 18 were considered opportunistic, 10 were not, and 4 were

not categorized because of insufficient data (J. Mills and D. Carroll, personal experience). There are two possible explanations for this pattern. It is possible that sylvatic species may harbor an equal number of zoonotic viruses but we may have selectively recognized those viruses associated with opportunistic, peridomestic species because those are the species most likely to come into contact with humans and to be associated with human disease. On the other hand, it is possible that there is something different about the (genetically determined) life history of opportunistic species that makes them more likely to have evolved and maintained relationships with zoonotic viruses (e.g., their relatively greater aggressiveness, high fecundity, or propensity to achieve very high population densities).

Behavioral factors

Certain specific behaviors have been shown to be associated with the transmission of hantaviruses and arenaviruses within host populations. Venereal transmission, which has been suggested for Machupo virus [22], implies a certain seasonality (assuming that breeding in host populations is seasonal) and thus predictable variation in risk for human populations. Transmission of some other arenaviruses (e.g., Junín virus) and hantaviruses in host populations seems to be associated with a different behavioral mechanism. Antibody in host populations is more common in males than in females, and is more common in larger, older animals [1, 5, 14, 29], implying horizontal transmission by a mechanism that favors males. Field studies have provided data that may identify that specific mechanism. Mammalogists frequently use the presence of scars as indicators of aggressive interactions among individuals. Field studies have shown that males more frequently have scars than do females and antibody-positive males are much more likely to have scars than are antibody negative males [14, 29]. This suggests that a frequent mechanism of transmission of these viruses in host populations is by fighting and inflicting bite wounds.

As might be expected from the hypothesized route of transmission, seroconversions to hantaviruses occur during the breeding season in many areas. In Arizona male brush mice seroconvert to Limestone Canyon virus throughout the breeding season, but only rarely in winter [1]. In high-altitude areas in Colorado, there is a second peak in seroconversions during mid winter [5]. This suggests a second behavioral mechanism of transmission, perhaps associated with communal nesting and mutual grooming during cold weather. An understanding of these different mechanisms of transmission is important if we are to develop accurate models of virus transmission and human risk.

A second behavioral characteristic that has important implications for human risk is habitat selection, which can be viewed on a regional scale, or on a micro (local) scale. On a regional scale, we found that deer mice were found in every major biome represented in the southwestern United States, from desert to alpine tundra. Furthermore, at least some deer mice infected with SNV were found in all of these habitat types. Nevertheless, the relative density of deer mice and especially the relative density of antibody-positive deer mice varied widely among

habitat types. The lowest densities and prevalences were found in the altitudinal and climatic extremes (desert and alpine tundra) and the highest densities and prevalences were found in the middle altitude habitats, such as pinyon-juniper woodland and great basin scrub [30]. Although I have placed this example in the behavioral category, much of the pattern may also be due to physiological tolerances. Regardless, knowledge of these differences allows a more accurate prediction of risk to humans living or traveling in various habitat types.

On a micro scale, habitat selection can be an important determinant of viral transmission among rodent hosts and from rodent hosts to humans. I have discussed the propensity of some rodents (such as deer mice) for peridomestic habitats, where they are more frequently antibody-positive than they are in sylvan habitats. Argentine hemorrhagic fever (AHF), caused by Junín virus, is associated with farming activities in rural Argentina. Descriptions of the epidemiology of AHF have stated that farmers are infected while working in crop fields [6]. However, during a three-year longitudinal study, we found that the reservoir for Junín virus, *Calomys musculinus*, was largely restricted to the more stable weedy roadsides and fencerows between crop fields. Its congener, *C. laucha* was frequently found in crop fields [29]. This pattern of habitat partitioning between the two closely related species may be very important epidemiologically. It may help explain lack of infection with Junín virus in *C. laucha*, it suggests a specific high-risk habitat for contracting AHF, and it suggests a potential mitigation practice – cutting or burning the weeds along the roadsides and fence lines that separate crop fields.

Physiological regulators

As mentioned above, infections with many hantaviruses and some arenaviruses are more frequent in male mice, and seroconversions are generally more frequent in the breeding season. Transmission of virus within host populations is also greater under more crowded conditions [16, 29, 31]. As explained above, these characteristics are due, at least in part, to behavioral factors. On the other hand, evidence is accumulating that physiological mechanisms might also contribute to this pattern of infection. Stress, associated with crowding, has been associated with immunosuppression in many animals, including some hantavirus host species (*Microtus*, *Rattus*, *Clethrionomys glareolus*) [11, 13, 24]. Increases in sex hormones, especially testosterone and corticosterone, have been clearly associated with immunosuppression in several species [2, 42]. Finally, the balance between type A and type B response to infection with Machupo virus was dose dependent [22]. It stands to reason, therefore, that the frequency of transmission and infection also might vary according to the degree of immunosuppression of the host.

Above, I suggested that the correlation between scars and infection status in hantavirus and arenavirus host rodents means that aggression leads to infection. Recent evidence suggests, however, that the cause and effect relationship between aggression and infection may not be as simple as it appears. Klein et al. [23] showed

that male rats in the chronic stage of infection with Seoul virus were more likely to attack intruders and they spent more time fighting with them than did uninfected males. Furthermore, aggressive males had more virus in tissues (including testes and adrenal glands) than did less aggressive males.

Many other physiological factors may be related to viral infection, viral shedding, and viral persistence. These include those related to social interactions, nutrition, environmental conditions (e.g., temperature and rainfall), intake of plant secondary chemicals, and even the pH or presence of protein in the urine (which may be influenced by diet). Our knowledge of these potential relationships is scant.

In summary, regulators of viral infection in natural hosts are numerous. Most of these regulators have been inadequately studied and remain poorly understood. These regulators do not act independently; their total effect varies according to their interactions with other regulators. Nevertheless continued research and improved understanding of these regulators is important. The better we understand these regulators, the better we will be able to predict changes in disease risk to human populations, develop effective intervention programs, and appropriately and most efficiently target these intervention efforts.

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Population dynamics of RNA viruses: the essential contribution of mutant spectra

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Summary. Cells and their viral and cellular parasites are genetically highly diverse, and their genomes contain signs of past and present variation and mobility. The great adaptive potential of viruses, conferred on them by high mutation rates and quasispecies dynamics, demands new strategies for viral disease prevention and control. This necessitates a more detailed knowledge of viral population structure and dynamics. Here we review studies with the important animal pathogen Foot-and-mouth disease virus (FMDV) that document modulating effects of the mutant spectra that compose viral populations. As a consequence of interactions within mutant spectra, enhanced mutagenesis may lead to viral extinction, and this is currently investigated as a new antiviral strategy, termed virus entry into error catastrophe.

Introduction

Genomics is unveiling a perplexing complexity of all differentiated genomes and their viral and cellular parasites. Complexity is presented in different flavors. The DNA of differentiated organisms includes mobile and retroviral-like elements or their relics, reflecting past (and probably present) dynamics of genetic modification and exchange within and between cells of the same and different organisms [for reviews see [3, 10, 53, 55]]. Genetic elements endowed at present (or in earlier stages of life) with mobility constitute about 40% of the human genome. Lateral (also termed horizontal) gene transfer, the process by which DNA from one organism captures DNA from another (distantly related) organism, is most frequent in prokaryotes, but also occurs in eukaryotes. The unicellular nature, spatial mobility, and presence of extrachromosomal genetic elements in most prokaryotes permit gene transfers via conjugation, fusion, assimilation, transduction, and transformation. Some of these processes are infrequent or inefficient in eukaryotes.

Viruses can also be regarded as agents of gene transfer between prokaryotic or eukaryotic cells [review in [7]]. Viruses spread differentially within tissues and organs of the same individual host. This is because different cell types do not express identical sets of macromolecules that can be used as viral receptors. This results in compartmentalization of susceptibility to virus infection. To achieve the transfer of foreign genetic material, a virus must mediate integration of DNA into the recipient chromosome. Integrative bacteriophages and retroviruses use DNA integration as essential steps in their life cycles [10, 13]. Retroviral-mediated gene transfer can have profound phenotypic consequences for the host, including a variety of disease manifestations [10, 53, 70].

In viruses, bacteria, and unicellular eukaryotic organisms, complexity is manifested in an extensive genetic heterogeneity at the population level, mediated by mutation, homologous and non-homologous recombination, and genome segment reassortment [6]. Both viruses and free-living cells generally have replication cycles which are faster than those of the organisms that they parasitize or infect. In the case of many viruses, and in particular RNA viruses, mutation rates are in the range of 10^{-3} to 10^{-5} mutations per nucleotide copied [8, 28]. The molecular basis of the error-prone replication of RNA viruses is the absence or low efficiency of both proofreading-repair activities in viral RNA replicases and retrotranscriptases, and post-replicative repair pathways [which act on DNA but not on RNA [6, 24]]. Both high mutation rates and rapid replication contribute to adaptability in changing environments, which also is supported by the selective advantage of mutator subsets of pathogenic bacteria [21, 42]. These features of pathogenic viruses and bacteria affect, in a decisive manner, strategies for disease prevention and control. For this reason, and to gain insight into the nature of RNA virus populations, our laboratory is carrying out studies of the important animal pathogen Foot-and-mouth disease virus (FMDV) [reviews in [63, 68]]. These studies are aimed at understanding virus survival and at defining sets of experimental conditions that can drive viral populations to extinction.

Quasispecies dynamics

Populations of RNA viruses are not defined genetic entities but are distributions of related, non-identical genomes termed viral quasispecies [22, 24, 27, 32–34, 40]. Because of high mutation rates, viral mutants are produced continuously during virus replication, and in such a way that any individual genomic sequence has a very fleeting existence. Thus the system is a highly dynamic mutant cloud whose fine composition is constantly modelled by the environment. A distinctive feature of RNA genetics is that the rapid response to an environmental change is directed by ensembles of genomes rather than by individual genomes whose behavior cannot be understood except as part of an ensemble [24, 33, 34]. The quasispecies theory was formulated as a general theory of molecular evolution [30, 31] but has been extended to describe the dynamics of finite populations of replicons subjected to environmental (fitness) variations [34, 74]. Because of its

emphasis on mutation, quasispecies theory has been instrumental in understanding the behaviour of RNA viruses, as compared with other formulations of Darwinian evolution [58].

Two consequences of quasispecies dynamics are the transition into error catastrophe when mutation rates exceed a threshold value, and the accumulation of mutations when viruses are subjected to repeated bottleneck events. A comparison of the response of FMDV to enhanced mutation rates and to bottleneck passages has provided new information on quasispecies dynamics, and the mechanisms of virus survival.

Crossing the error threshold

One of the most experiment-provoking outcomes of quasispecies theory, one presented with its earliest formulations [71], is the prediction that for any replication system there is a maximum error rate compatible with maintenance of the information encoded in the replicating genome [4, 35, 57]. This concept is represented by the following error threshold relationship:

$$\nu_{max} < \ln \sigma_o / (1 - \bar{q}) \quad (1)$$

in which ν_{max} is the maximum length (in the sense of information complexity) that can be maintained during replication, σ_o is a parameter that measures the superiority or selectivity of the master (dominant) sequence relative to its mutant spectrum, and $\bar{q}$ is the average copying fidelity; therefore, the average error rate is $1 - \bar{q}$. The expected number of errors introduced in a genome of length ν replicating with fidelity $\bar{q}$ is $\nu(1 - \bar{q})$; $\bar{q}$ is an average value for the entire genome, and different positions of a replicating genome may have higher ($q_h > \bar{q}$) or lower ($q_l < \bar{q}$) copying fidelity than the average.

This fundamental equation [35, 57, 71] indicates that when the error rate $(1 - \bar{q})$ increases ν_{max} decreases. Applied to viruses, this leads to the prediction that an increase in the mutation rate (the number of misincorporations occurring per nucleotide during template copying) should result in a transition from a productive infection (the genetic information is maintained) to an abortive infection (the genetic information is lost in a process akin to "melting" in physics). A relevant consideration (which we owe to an insightful discussion with Profs. M. Eigen and C. Biebricher) is in place here. Depending on how many sites belong to the q_h or q_l category (sites of the genome showing higher or lower fidelity than the average) and where they are in the viral genome, the position of the error threshold for two virus-host-systems may differ despite the two systems sharing the same $\bar{q}$ (Fig. 1).

Experiments that showed that increasing mutation rates decreased viral infectivity were first carried out by J. J. Holland and his associates [46] working with a vesicular stomatitis virus (VSV) and a poliovirus, using a number of mutagenic agents [including the base analog 5-fluorouracil (FU) and 5-azacytidine (AZC)] to increase the mutation rate during viral replication. Following these initial observations, virus extinction associated with enhanced

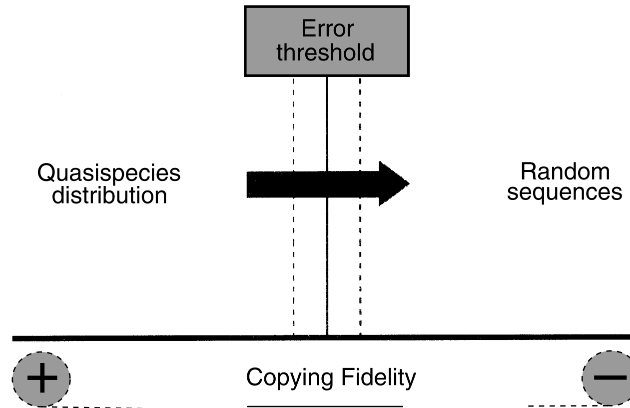


Fig. 1. Scheme of the transition into error catastrophe of a replicating system. Quasispecies distributions exist within a range of copying fidelities. Crossing the error threshold (large arrow) results in "melting" of the genetic information, that can be represented by a transition from an organized mutant spectrum into random nucleotide sequences. For a given genetic complexity, the position of the error threshold may vary depending on the types of mutations produced by a mutagenic agent, in relation to the types of mutations needed to affect essential viral functions. This is represented schematically by vertical discontinuous lines around the position of the error threshold given by an average copying fidelity (see Eq. (1) and text)

mutagenesis has been documented with a variety of virus-host systems [7, 15–18, 45, 47, 50, 51, 64] including our own work with FMDV [general reviews in [26] and [35]].

Two salient findings in this field of research have been the recognition that the nucleoside analogue ribavirin(1-β-D-ribofuranosyl-1,2,3-triazole-3-carboxamide) in some virus-host systems can exert its antiviral activity via enhanced mutagenesis [2, 16–18, 44, 52, 73]. Ribavirin is a licensed antiviral agent used to treat several important human infections such as with hepatitis C virus (HCV), often in combination with interferon-α [67]. A mutagenesis-based action of ribavirin *in vivo* is supported by the isolation, from ribavirin-treated patients, of ribavirin-resistant mutants that map in the HCV polymerase [75]. A ribavirin-resistant poliovirus mutant that confers altered fidelity to the poliovirus polymerase has also been described [61]. Therefore, it is possible that in certain cases ribavirin has exerted its antiviral activity *in vivo* according to the principles of virus entry into error catastrophe.

The second salient finding was by de la Torre and associates, who showed that pre-treatment of mice with FU prevented the establishment of a persistent lymphocytic choriomeningitis virus infection [63a]. This constitutes a proof of principle of the feasibility of a lethal mutagenesis-based antiviral approach *in vivo*. Together with the increasing number of significant results with ribavirin *in vivo*, they encourage further exploration of error catastrophe as an antiviral strategy.

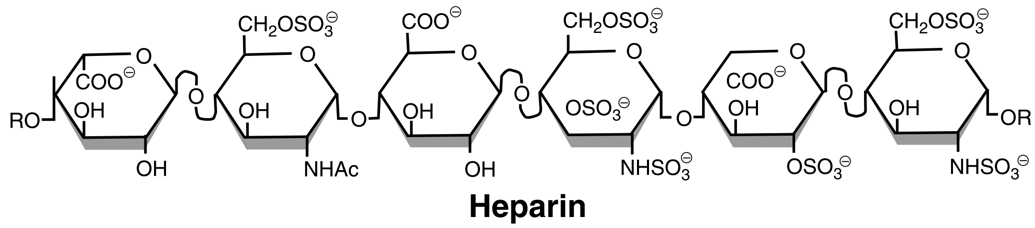
Error catastrophe of FMDV

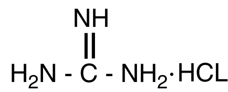
Extinction of FMDV by enhanced mutagenesis has been studied in cell culture using the mutagenic agents FU, AZC and ribavirin, alone or in combination with the antiviral inhibitors guanidine, heparin and mycophenolic acid (Fig. 2). During cytolytic infections in cell culture, low viral load and low viral fitness favoured extinction of FMDV by FU and AZC [59, 65]. The effect of fitness can be interpreted as a lower value of the superiority of the master sequence (σ_o in Eq. (1)) and, therefore, a decrease in ν_{max}. FMDV populations in their way towards extinction showed no mutations in the consensus sequence, but displayed increases in the complexity of the mutant spectra, as quantified by the mutation frequency and Shannon entropy [[43a, 59, 66] Gonzalez-Lopez et al., submitted for publication]. Remarkably, the maximum increases of complexity were quantified for the FMDV RNA-dependent RNA polymerase (3D) which is very conserved in FMDV. This probably reflects an uncontrollable accumulation of mutations in essential genes, which pushes the virus towards the error threshold. The recent elucidation of the three-dimensional structure of the FMDV RNA polymerase (3D), isolated and in a complex with template primer [39], together with multiple altered polymerases identified in mutagenized FMDV populations [2, 65, 66], may help in defining the molecular basis of FMDV extinction. Studies with several mutant polymerases are now in progress.

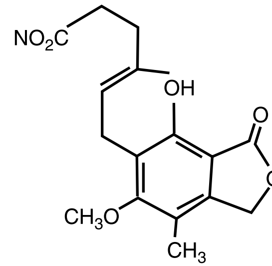
Ribavirin can cure persistently infected BHK-21 cells of the resident FMDV [19]. Ribavirin may exert its antiviral activity through several mechanisms, in addition to a direct mutagenic activity [67]. One of them is through a competitive inhibition of the enzyme inosine monophosphate dehydrogenase (IMPDH; EC 1.1.1.205) resulting in a reduction of the intracellular concentration of guanine nucleotides [69]. By comparing the activity of ribavirin with that of mycophenolic acid (another inhibitor of IMPDH) on nucleotide pools and on the complexity of persistent FMDV, it was concluded that the main mechanism by which ribavirin eliminated FMDV from carrier cells was direct, enhanced mutagenesis [2].

Consistent with the fact that low viral loads favoured extinction of FMDV during cytolytic infections in cell culture, combinations of the mutagenic agent FU with the inhibitors guanidine and heparin were more efficient than FU alone in driving FMDV to extinction [59, 60]. In particular, FMDVs showing high relative fitness required the combination of FU with the two inhibitors for a systematic extinction of FMDV after a few passages, in parallel infection series. When no extinction was achieved, selected inhibitor-escape mutants of FMDV allowed virus survival despite the presence of the mutagenic agent [60]. Therefore, in any antiviral design based on error catastrophe using combinations of mutagenic agents and antiviral inhibitors, it will become essential to ensure a sufficient mutagenic and inhibitory presence at all sites where viral replication takes place, to avoid selection of extinction-escape mutants.

Selection of inhibitor-resistant, mutant viruses is a common cause of failure of antiviral treatments [23, 25, 54, 62]. An advantage of error catastrophe versus







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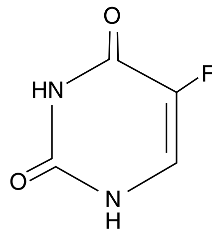

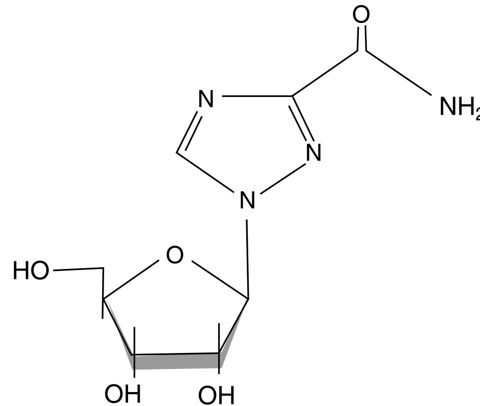

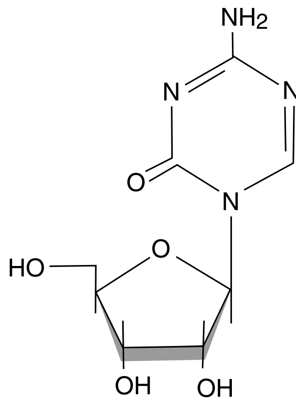

b

Fig. 2. Structure of the inhibitors (**a**) and the mutagenic agents (**b**) used in the studies on FMDV transition into error catastrophe

a classical approach based on administration of combinations of inhibitors alone, could be provided by the suppressive effect of a highly mutated spectrum of mutants on residual infectivity. Suppression of mutant spectra on higher fitness virus or on specific variant virus types was previously documented with VSV [20], with poliovirus vaccines containing virulent forms as minority components [12], with antigenic variants of FMDV [9] and with variants of LCMV with different potential to induce a growth hormone deficiency syndrome in mice [72]. RNA cotransfection experiments have shown that mutated, preextinction RNA is able to interfere with residual infectious RNA, resulting in a delayed viral production [43]. The interference was exerted specifically by high molar mass preextinction RNA, and was not the result of induction of interferon or other nonspecific antiviral responses. This type of interference is probably mediated by abnormal expression of normal and aberrant FMDV proteins that cause local "intracellular chaos" that impedes completion of the virus replication cycle. As an antiviral strategy, this may represent an advantage of virus entry into error catastrophe versus inhibition [43].

Resistance to extinction despite accumulation of mutations upon serial bottleneck passages of FMDV

Efficient extinction of FMDV by combinations of mutagens and inhibitors establishes an interesting contrast with resistance to extinction of FMDV clones subjected to repeated bottleneck events, experimentally achieved with serial plaque-to-plaque transfers [11, 29, 36, 37, 77, 78]. Fitness loss associated with serial bottleneck passages constitutes experimental evidence for the operation of Muller's ratchet [56], a theoretical concept predicting that asexual populations of organisms tend to accumulate deleterious mutations unless compensatory mechanisms such as sex or recombination intervene.

The experiments with FMDV have documented that, indeed, fitness loss occurs as a result of plaque-to-plaque transfers but that the decrease, rather than being uniform, follows a complex pattern [38, 48, 49]. During the first 20 to 40 transfers, relative fitness fluctuated around an average value that decreased in a nearly exponential manner. In the following transfers, the amplitude of the fluctuations increased, and the average fitness values remained constant. This fluctuating, biphasic pattern of fitness decrease was robust since it has been invariably seen with a number of independent passage series of different FMDV clones [38, 49]. The result of this behaviour is a remarkable resistance to extinction despite a linear accumulation of mutations, at a rate of about 0.25 mutations in the consensus sequence per plaque transfer (Table 1).

In the course of plaque transfers, the types of mutations and their distribution along the FMDV genomes were very different from those observed in other FMDV populations evolved under different passage regimens [38]. In particular several, but not all, clones subjected to plaque-to-plaque transfers generated an internal oligoadenylate tract as an extension of four adenylate residues that precede the second functional AUG in the FMDV genome [5, 36]. After about 190

Table 1. Comparison of FMDV populations subjected to enhanced mutagenesis and to serial bottleneck transfers

	Mutant spectrum	Consensus sequence	Outcome
Enhanced mutagenesis	2- to 10-fold increase in complexity	No change	Frequent extinction
Bottleneck transfers	Heterogeneous (not quantified)	Linear accumulation of mutations (0.28 per genome and plaque transfer)	Infrequent extinction

Based in [2, 5, 36, 38, 43a, 59, 60, 65, 66]

plaque-to-plaque transfers, some extinction events have been documented for clones with the internal oligoadenylate when the consensus sequence reached a mutation frequency of 6.5×10^{-3} mutations per nucleotide relative to the parental clone (Escarmís et al., unpublished results).

A numerical model was developed that explains both the biphasic kinetics of fitness loss and the fluctuation of fitness values in the course of the serial plaque transfers [49]. Indeed, determination of the genomic changes that accompany fitness gains of FMDV clones has documented very few true reversions, and several additional mutations [[37]; (Escarmís et al., unpublished results)]. Therefore, a considerable resistance of low fitness clones to extinction, despite accumulation of mutations, appears to be mediated by the occurrence at low frequency of advantageous mutations, those presumably selected during plaque development. According to this model, survival is the result of the virus behaving as a spectrum of mutants: many individuals are extinguished and a minority of individuals acquiring compensatory mutations together permit survival. A calculation of the ratio of total genomic RNA molecules to infectious units in individual plaques gave a wide range of 10^4 to 10^7, depending on the fitness of the virus in the plaque. Remarkably, when a stationary phase of fitness values was reached, there was a persistent drift in sequence space driven by heterogeneous viral populations found within individual viral plaques [[38]; (Escarmís et al., unpublished results)]. Again, minorities in mutant spectra permit survival of the virus as a population.

Mutations for survival or for extinction

A comparison of the features of FMDV populations on their way to extinction by enhanced mutagenesis, and populations subjected to plaque transfers and which elude extinction (Table 1), provides new insights into viral population dynamics. Mutagenesis-driven extinction of FMDV appears to be favored when the size of the viral population is small, the fitness of the virus low, and the mutational input does not allow for selection of compensatory mutations. In contrast, the tolerance of FMDV to accept mutations while remaining functional after repeated bottleneck passages appears to be based on occurrence of advantageous mutations which

in minority subpopulations rescue viral infectivity. The key role of the mutant spectrum (neglected when evolutionary events are monitored exclusively with consensus sequences!) is manifested in both cases. In the transition to extinction, the "melting" of information is reflected in increasing complexity of the mutant spectrum. In survival during serial bottlenecks, the mutant spectrum is the source of genome subpopulations able to be rescued from excess mutations by critical, compensatory mutations.

The contribution of mutant spectra to the limitation of expression of individual variants, or in driving the virus to extinction, can be regarded as an extension to a single virus of the interfering interactions which have been documented to occur between very different viruses or between one virus type and its defective-interfering particles [[41]; reviews in [1, 14, 27, 76]]. A subset of mutants of the mutant spectra may contribute altered *cis*-acting signals and *trans*-acting gene products, which collectively may act similarly to a dominant-negative mutant. According to this model, interference should be favoured by the multifunctional nature of many viral proteins (particularly in RNA viruses with a compact genetic information) and the extent of the interference should depend on the mutational load in the components of the mutant spectra. Experiments are now in progress to test this model.

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Control of arbovirus diseases: is the vector the weak link?

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Summary. Arthropod-borne virus (arbovirus) diseases (ABVDs) remain major threats to human health and well-being and, as an epidemiologic group, inflict an unacceptable health and economic burden on humans and animals, including livestock. The developed world has been fortunate to have escaped much of the burden that arboviruses and their arthropod vectors inflict on humans in disease endemic countries, but the introduction and rapid spread of West Nile virus in the Western Hemisphere demonstrated that we can no longer be complacent in the face of these emerging and resurging vector-borne diseases. Unfortunately, as the burdens and threats of ABVDs have increased, the U.S. and international public health capacity to address them has decreased. Vaccines are not available for most of these agents. Previously successful strategies to control ABVDs emphasized vector control, but source reduction and vector control strategies using pesticides have not been sustainable. New insights into vector biology and vector pathogen interactions, and the novel targets that likely will be forthcoming in the vector post-genomics era, provide new targets and opportunities for vector control and disease reduction programs. These findings and approaches must be incorporated into existing strategies if we are to control these important pathogens.

Resurgence and emergence of arbovirus diseases

In the 20th century, extraordinary advances were made in the diagnosis, treatment, and control of many infectious diseases. These successes were not uniform and, unfortunately, the medical, veterinary, and economic importance of many ABVDs has continued and indeed increased. Dengue (DEN) exemplifies the problem; more than 2.5 billion people are at risk for dengue virus infection, and 100 million cases are estimated to occur annually. This is especially disconcerting because DEN was controlled to a degree in some parts of the world, but now is resurgent throughout the world. Indeed, the incidence of life-threatening dengue hemorrhagic fever and dengue shock syndrome (DHF-DSS) has increased rapidly throughout the tropics over the past 20 years. Epidemic DEN and DHF-DSS have emerged as major public health problems in the Americas, mirroring what happened in

Southeast Asia several decades ago [33, 35]. Other ABVDs have emerged in or trafficked to new or previously endemic areas, e.g., Japanese encephalitis, West Nile encephalitis and Rift Valley fever (RVF), resulting in significant morbidity and mortality [36, 59]. The burdens imposed by DEN and other ABVDs can be impediments to social and economic development in areas of the world which are least able to afford them. The emergence of epidemic DEN and DHF-DSS has resulted in its inclusion as one of the 10 diseases targeted by the W.H.O. for special control programs because of their overwhelming public health and socioeconomic importance (WWW.WHO/INT/TDR/).

Arboviruses also cause significant economic impact in agriculture. Bluetongue and vesicular stomatitis are classified as List A diseases by the International Office of Epizootics. Worldwide economic losses due to bluetongue are estimated at $3,000,000,000 per year, principally due to non-tariff barriers to international trade. Vesicular stomatitis epizootics in the western U.S. in 1995 and 1997 resulted in losses of ca. $50,000,000. International trade agreements (e.g., GATT and NAFTA) and globalization provide opportunities as well as threats for agriculture. Inadvertent importation of vectors and pathogens would seem to be the inevitable result of increased movement of animals and products, and of increased trade in general [22]. Introduction of RVFV into a new area could result in overwhelming morbidity and mortality in livestock, as occurred when the virus emerged in Egypt [60].

Finally, a number of arboviruses are potential bioterroism agents and several are known to have been weaponized. A natural (or purposeful) introduction of a bioterrorism agent, such as RVFV, would have enormous agricultural and public health consequences.

The emergence of West Nile virus (WNV) in New York in 1999 [55] clearly demonstrated the vulnerability of the United States to emerging diseases, whether resulting from natural or purposeful events. Such emergences, and the resurgence of diseases such as DEN and yellow fever (YF) highlight the deficits in human resources and infrastructure needed to address ABVDs, and the critical needs to augment our armamentarium and approaches to control of these diseases.

Factors contributing to the resurgence of arbovirus diseases

Many factors have conditioned the resurgence of ABVDs. These factors have been discussed in great detail elsewhere [9, 33, 36, 85], but some will be discussed here briefly, to provide insight into the difficulties, needs, and complexities of controlling ABVDs.

Lack of vaccines and problems with vaccine deployment

Developing efficacious vaccines and deploying them effectively has proven to be difficult. For example, to develop an efficacious vaccine for DEN, a vaccine must simultaneously immunize against all four serotypes of the virus in order to preclude the possibility of immune enhancement [39]. Novel strategies for preparing DEN vaccines are being investigated. These offer promise [54]. However, even if an inexpensive and efficacious vaccine for DEN should become available, the

lack of public health infrastructure in areas with the greatest need might well curtail application. For example, there has been an efficacious and inexpensive vaccine for YF for decades, yet this virus continues to cause significant mortality in humans in Africa and South America. Frighteningly, the number of available doses of YF vaccine is limited, and would not be sufficient to vaccinate those at risk should YF emerge in an urban cycle in a large South American or African city or in Asia; the latter occurrence would result in a major public health catastrophe [62]. Hopefully, the new interest by government agencies and private foundations in addressing the terrible burden of infectious diseases in the developing world may result in the development of new vaccines and of effective systems to deploy them.

Pesticide resistance in vector populations and aversion to pesticide usage

Pesticide resistance in vectors is also emerging as a factor in controlling malaria [42]. The number of pesticides available for mosquito control is severely limited [41]. Numerous studies are now documenting resistance in *Aedes aegypti* to commonly used pesticides, potentially removing these from the armamentarium used by mosquito control officials to control DEN. Increasing resistance to temephos, which is widely used for control of *Ae. aegypti*, is of great concern [e.g. 56]. Similarly, pesticide resistance is widespread and prevalent in *Culex* populations, potentially precluding chemical control of these vectors in impending or ongoing epidemics [e.g. 15]. Clearly, pesticide resistance in vectors is a major problem and one that will undoubtedly worsen without the development of new, environmentally sensitive pesticides.

Societal aversion to the use of pesticides, based on perceived environmental and health effects, is of great concern. This has led to resistance to pesticide application even in the face of ongoing epidemics, such as WNV epidemics in New York and Colorado, and to the removal of pesticides from control programs. In this regard, the discovery and subsequent use of DDT to control vector-borne diseases was a major achievement in public health [6, 78]. However, indiscriminant DDT usage associated with agricultural practices led to detrimental effects in non-target organisms, and DDT was banned even for public health use in indoor residual spraying (IRS) programs. New pesticides have proven to be more expensive, less stable, and less efficacious than DDT. The widespread termination of DDT usage coincided with a resurgence in DEN, malaria, and other diseases that are transmitted principally indoors by endophilic and endophagic vectors [6, 33, 78]. Clearly, development of environmentally sensitive insecticides with the efficacy of DDT is a public health imperative.

Erosion of public health infrastructure and medical entomology expertise

Surveillance and control programs for control of ABVDs are especially vulnerable to reductions or elimination when budget shortfalls occur and when ABVD activity

is incorrectly perceived to be controlled. The consequences of such reductions can be dramatic. For example, in the 1950s and 1960s the Pan American Health Organization and participating Western Hemisphere countries established a program to control *Ae. aegypti* to preclude the emergence of sylvatic YF into urban populations [33, 35]. Overall, the program was quite effective, but success led to demise of the programs, and the resources to support these efforts were shifted to other priorities. Now *Ae. aegypti* is essentially hyperabundant throughout much of tropical and sub-tropical America, and all 4 DEN virus serotypes, including virulent Asian genotypes, are co-circulating in Latin America [9, 35]. Mosquito abundance and intensive virus transmission have resulted in a state of DEN hyperendemicity, resulting in the emergence of DHF-DSS as a major public health problem in the Americas. In addition, YFV has recently caused epidemics in South America [36, 62], and with *Ae. aegypti* resurgent in metropolitan areas in the Americas, it seems to be only a question of when, not if, urban YF will re-emerge to once again wreak havoc on humans.

The world wide reduction in the numbers of medical entomologists, vector biologists, and vector control personnel has contributed to lack of control of ABVDs. Indeed, it was difficult to identify local medical entomologists, vector biologists, and arbovirologists to respond to the WNV emergency in the initially affected states in the U.S. The public health implications of the loss of expertise in these areas were first described in a U.S. National Academy of Sciences Report [23], and the critical needs in this area have been reasserted in subsequent publications [85, 86]. Clearly, rebuilding the national and international expertise in medical entomology and related disciplines is critical.

Poverty and social inequalities and the throw away society

The problems in controlling ABVDs are exacerbated greatly by socioeconomic and behavioral issues. Poverty and social inequalities are major factors in the resurgence of ABVDs, especially in tropical regions. One recent epidemiological investigation of DEN in "sister"cities in Mexico and Texas illustrates this very well [76]. In the U.S. city there was very little dengue, but *Ae. aegypti* was relatively abundant. In contrast, in the Mexican City, there was much DEN, but mosquito control was more effective than in the U.S. city. The investigators attributed this to numerous factors, one of the most important being the quality of housing and life style. From this and other studies, it is clear that poverty is directly linked to the dramatic growth in human populations, unplanned urbanization, and movement of humans, all of which can condition the resurgence and emergence of ABVDs [36, 85].

Explosive population growth is a major determinant of the emergence and resurgence of dengue and other ABVDs. Dramatic increases in urbanization are frequently associated with little, poor, or no civic planning and sanitation may be limited or nonexistent [33, 36]. Many newly urbanized areas do not have piped water, and stored water provides plentiful breeding sites for *Aedes* and *Culex* vectors. These areas typically have minimal refuse removal, and in the "throw

away society" this has major implications in ABVD control. Even in the poorest of societies, the proliferation of bottles, cans, old tires, etc. in the environment provide a plethora of breeding sites for container breeding mosquitoes and dramatically complicate source reduction and larvicide control programs [85]. Population growth and other socioeconomic factors also frequently result in humans migrating into undeveloped areas. There they impinge upon sylvatic (jungle) cycles of ABVDs (e.g., YFV), potentially leading to the emergence of new diseases.

Rapid dissemination of pathogens and vectors in the global economy has contributed greatly to the resurgence and emergence of ABVDs. The global economy, which is predicated upon commerce and rapid and efficient transport of goods and people, provides unprecedented capability for emergence and rapid dissemination of pathogens and their vectors throughout the world [85]. Recent emergence of WNV in the New World [79] is testimony to the ability of ABVDs to traffic rapidly into new areas. The many reports of airport and railroad malaria also illustrate the continual trafficking of pathogens [58].

Vectors themselves can also traffic to and become established in new areas. *Aedes albopictus*, the Asian tiger mosquito, and *Ae. japonicus* presumably entered the U.S. via shipping [26, 63]. *Aedes* spp. eggs can easily be transported in tires and other containers to new areas and may hatch upon exposure to water in new settings. Adult mosquitoes can be spread much more quickly throughout the world in airplanes [58]. Indeed, such transport has been postulated as a mechanism for the rapid dissemination of a pesticide resistance mutation in *Culex pipiens* populations throughout the world [75].

Lack of new targets and approaches to control vectors

Complicating control of ABVDs has been a lack of new targets and approaches for control of vectors. Indeed, the vector has frequently been viewed as a black box; pathogens entered the box and subsequently were transmitted, but little was known about the intervening events [12]. In the last decade, there has been a revolution in vector biology. Much of this revolution can be attributed to the application of modern molecular techniques to address issues involving vectors. This effort is certainly helping to remove the view of the vector as a black box and is providing exciting new information and targets for potential control efforts. Some of the exciting advances resulting from this revolution follow. The emphasis will be on, but not limited to, *Ae. aegypti* and DEN.

Vector genomics/genetics

The advent of PCR had a major impact on vector biology [11]. This technology permitted the assay of multiple markers from the small amount of DNA that can be obtained from individual organisms, and even from an organ of an individual. PCR-based approaches have greatly facilitated studies of the population genetics of vectors, mapping of loci that condition vector competence and vectorial capacity, development of molecular markers for studies of vector taxonomy and

systematics, production of molecular assays for determination of pesticide resistance, and surveillance for pathogens in vectors. This remarkable progress culminated in the publication of the genomic sequence of *An. gambiae* in 2002 [47]. *In silico* approaches to identify vector genes began with the publication of the *Drosophila melanogaster* genome sequence and exploded with the publication of the *An. gambiae* genome sequence. An *Ae. aegypti* genome project is underway, and comparative studies of the genomes are underway [13, 82]. Other vector genome projects are in the pipeline. The post-genomics era in vector biology offers great promise for identifying new targets and approaches for control of vectors.

Molecular markers provide unparalleled insight into the breeding structure and population genetics of *Ae. aegypti*; in Mexico three distinct genetic groups of *Ae. aegypti* have been identified, with barriers to gene flow between the groups [32]. Populations on the two sides of a barrier may not only differ genetically but also in vector competence [10]. Molecular markers have proven to be extremely robust for taxonomic investigations of vectors of arboviruses [5, 27, 65]. Molecular linkage maps provide unprecedented ability to map and identify candidate genes that condition phenotypes of interest [12, 14, 81]. Mosquito genomics including microarray technology has revolutionized identification of mosquito genes and groups of genes that respond to pathogen infection of epidemiologically significant target organs. The approach is particularly powerful for comparing the genetic responses in competent and incompetent vectors and for identifying genes that may be exploited to interrupt pathogen transmission.

Characterizing gene function

Identifying candidate genes for control is only the first step; gene function must be demonstrated. Even in the recent past, this could be a Herculean task when working with vectors. New tools for gene function characterization include virus expression systems, RNA interference (RNAi), and transformation. Development of Sindbis virus (family *Togaviridae*) double subgenomic (dsSIN) expression vectors was the first step in this regard [69, 71]. Sindbis virus is an arbovirus, and thus has the ability to infect and replicate for the life of an infected vector. Infectious clones of Sindbis virus can be engineered with a second subgenomic promoter to express robustly a gene or sequence to silence a gene of interest in vectors or other arthropods [30, 68]. This powerful tool has been used to determine potential candidate viral sequences for engineering resistance to DEN viruses, La Crosse virus, and YFV infections in vectors [1, 44, 70, 74], to silence and characterize gene function in arbovirus vectors [50, 83], and to express genes of interest in vectors [43, 45, 72]. The mechanism of dsSIN transducing virus gene silencing has been shown to be RNAi [80]. RNAi can be used to silence genes of interest for functional analysis of *Ae. aegypti* genes and for arbovirus-vector interactions in vitro and in vivo [2, 7, 16, 84, 91]. Major breakthroughs have been made in genetic transformation of *Ae. aegypti* and *Culex quinquefasciatus*, providing unprecedented capability for gene characterization in these arbovirus

vectors [3, 19, 20, 34, 49, 53, 66]. Major advances are being made in developing and exploiting transformation systems for characterization of mosquito genes [67], including development of promoters for expressing genes of interest in specific tissues and at specific times [52]. Although not nearly as robust as P-element transformation for *Drosophila melanogaster*, this technology offers great potential for gene characterization.

Vector biology and vector/pathogen interactions

The ability to identify genes of interest and availability of the tools to functionally characterize them have led to an explosion of information about fundamental elements of vector biology that condition vectorial capacity. Such information has potential for manipulation of vectors and control of pathogen transmission. A few of these will be discussed briefly.

Attractants and repellents

Development of new repellents and attractants for vectors is critical. Molecular biological and now *in silico* approaches are providing exciting new insight into the molecular biology of olfaction in vectors. Indeed, much information is forthcoming concerning odorant binding proteins and arrestins that are key components of the behavioral response. For example, a number of *Anopheles gambiae* odorant receptors (OR) and arrestins, which are key determinants of host seeking in vectors, have been identified [28, 61, 73]. One OR (AGOR1) was demonstrated to be expressed only in adult females and is an OR that responds to a component of human sweat [38]. Information generated from such studies offers great potential for development of new repellents and attractants to control vector populations.

RNAi

RNAi is not only a tool for gene characterization, it is also an important component of the innate immune response of vectors [46, 80]. Vectors mount an RNAi response upon infection with RNA viruses. Initial in vitro studies demonstrated that mosquito cells, which had been stably transformed with constructs expressing DENV sequences in sense, antisense, or in a fold-back sequence, were resistant to DENV, with the most resistant lines being ones that had been transformed with the fold-back construct [2]. The expressed fold back sequence presents a double-stranded RNA target that facilitates Dicer recognition of the aberrant RNA species [80]. In addition, infection of mosquito cells or midguts with Sindbis virus, DEN virus (family *Flaviviridae*), or La Crosse virus (family *Bunyaviridae*) induces an RNAi response, as evidenced by the accumulation of small interfereing RNA species. Finally, silencing of certain RNAi response genes makes resistant vectors susceptible to infection with arboviruses (Keene, K., unpublished data). Obviously, upregulating innate immune responses of mosquitoes could become a powerful tool for reducing vector competence.

Autogeny

The holy grail of vector molecular biology has been determining the molecular bases of anautogeny. Induction of autogeny (no blood feeding necessary for a gonadotrophic cycle) in mosquitoes would be the ultimate control strategy. They would no longer need to seek blood meals and thus would not transmit pathogens. Anautogeny refers to the need by the mosquito for a blood meal in order to undergo a gonadotrophic cycle. The blood meal is necessary for the initiation of vitellogenesis and egg development. Anautogeny provides the evolutionary drive for vector/host interactions and thus drives the acquisition and transmission of pathogens. Great strides have been made in understanding the molecular biology of anautogeny. Prior to blood feeding, anautogenous mosquitoes maintain their reproductive system in a state of arrest. A GATA repressor in *Ae. aegypti* that inhibits expression of the yolk precursor protein (YPP) has been identified in *Ae. aegypti* [7]. Silencing this GATAr with a Sindbis virus expression system results in abundant expression of YPP. Importantly, amino acids in the blood meal naturally derepress the GATAr via the TOR kinase pathway, thereby leading to a gonadotrophic cycle [40]. This elegant work, and similar investigations of other molecular determinants of vectorial capacity, could lead to novel ways to control vector-borne diseases.

Vector-pathogen interactions

New approaches and tools also have revealed exciting new information concerning the molecular determinants of vector-pathogen interactions. Studies are identifying genes that condition important vector-pathogen phenotypes and thus are targets for strategies to interrupt pathogen transmission [12]. For example a molecular linkage map was used to identify quantitative trait loci that condition DEN virus infection of *Ae. aegypti*. Provocatively, midgut early and abundant trypsins were identified as candidate molecules in this interaction [14]. Inhibition of early trypsin with a dsSIN virus expression system resulted in a much lower DEN infection rate in vectors. The post-genomics era in *Ae. aegypti* will undoubtedly provide a plethora of new candidate genes for control.

There is much value added to the identification of genes that condition important vector phenotypes; polymorphisms in genes that condition vectorial capacity can be factored into risk assessment models and surveillance systems for predicting the emergence of diseases.

Genetic manipulation of vector populations

Systems to genetically manipulate vectors for gene characterization (e.g., transposable elements and transducing viruses) as well as arthropod symbionts (e.g., *Wolbachia*, *Rhodococcus*) are also being investigated as drive or delivery mechanisms to manipulate genetically vector populations to reduce or stop pathogen transmission [9]. For example, *Ae. aegypti* innate immune genes could theoretically be induced in a vector population using a transposable element or

transducing virus drive mechanism expressing a DENV sequence. Such technologies and strategies could provide another critical tool to augment integrated pest management approaches to control vector populations and to interrupt pathogen transmission. There is already considerable laboratory proof of concept that molecular manipulation of vectors can make them resistant to pathogen infection [8, 48, 70].

Proof of concept that mosquitoes could be molecularly manipulated to make them incompetent vectors was first demonstrated with arboviruses. Inoculation of mosquitoes with dsSindbis viruses expressing genomic sequences of La Crosse and DEN viruses induced nonpermissiveness in *Ae. triseriatus* and *Ae. aegypti* to the respective viruses [70, 74]. Paratransgenic strategies have also been developed and proposed for control of Chagas disease [8]. Symbiotic bacteria of triatomine bugs can be engineered to express in the vector genes that are detrimental to the parasite, and that inhibit parasite transmission [24]. Mosquitoes also can be engineered to express molecules that impair the ability of parasites to productively infect vectors. For example, a phage display library approach was used to identify a unique peptide (designated SM1) that bound to midgut and salivary glands of mosquitoes [31]. Transgenic *An. stephensi* mosquitoes expressing the peptide were resistant to the parasite *Plasmodium berghei* and inefficiently transmitted it to mice [48]. Similar strategies could be developed to stop arbovirus infection of and transmission by mosquitoes.

The use of genetically modified mosquitoes to interrupt the transmission of pathogens, such as DEN viruses and malaria parasites, provides novel approaches to complement existing approaches for control of VBDs [17, 21, 77]. Obviously, development of pathogen resistant vectors is only the beginning. Numerous and critically important scientific, ethical, safety, and regulatory issues will need to be addressed and the risks and benefits of such an approach will need to be critically assessed before such an approach could and should be used [4, 9].

Novel immunization strategies

Insights into the molecular biology of vectors have provided exciting new opportunities for the development of transmission blocking new vaccines against ABVDs [18]. Landmark studies by Titus and Ribeiro [87] revealed that sandfly saliva greatly potentiates leishmania infection of hosts. Targeting the vector saliva to control pathogen transmission potentially precludes problems with developing vaccines against the parasite and could provide broad-spectrum protection for multiple strains of pathogens. Vertebrate hosts can be immunized with vector saliva or salivary proteins to protect them from infection with leishmania parasites [51, 64]. Salivary potentiation also occurs for arboviruses vectored by flies and ticks [25, 37, 57], and immunization against dipteran salivary gland proteins could theoretically protect against infection with multiple arboviruses. This approach is very exciting, and new salivary gland genomic/proteomic approaches are revealing a plethora of new vaccine candidates [88, 89]. Vector-killing vaccines also offer potential for vector control [29]. In this approach, vectors feeding upon

hosts immunized with "hidden" antigens of vectors are killed [90]. Vector-killing vaccines were thought not to be candidates for control of mosquito-borne diseases because of the brief feeding time of the vector. However, survivorship of *An. gambiae* mosquitoes fed on mice that had been immunized with a cDNA of the *An. gambiae* mucin gene was dramatically less than that of mosquitoes fed on control mice. Interestingly, the mouse cell-mediated immunity response conditioned the vector killing [29]. Molecular biologic, genomic and proteomic approaches are identifying a plethora of vector proteins that condition pathogen infection of and transmission by vectors as well as vector survival. Immunization of the host to target specific vector genes or to perturb a specific vector-pathogen interaction could be a powerful approach to stop arbovirus transmission.

Translation of emerging knowledge of vector biology and vector-pathogen interactions

The major advances in understanding the biology and molecular biology of vectors and vector-pathogen interactions provide promise for the development of new targets and opportunities for control, especially in the post-genomics era of vector biology. While much of the discussion and research to exploit this knowledge has focused upon genetic manipulation of vector populations, application of such approaches is far in the future. It is important to note that such information could also be exploited to control ABVDs by delivery of effector systems in other control approaches. For example, effector molecules could be delivered in stations baited with attractants or incorporated into insecticide treated materials, such as bed nets and curtains, or even in sprays intended to manipulate or kill vectors. Such approaches could be integrated into existing control programs relatively straightforwardly.

Discussion

Overall, there is considerable excitement in vector biology. Progress in understanding the molecular biology of vectors has been extraordinary. The mandate is to translate this explosion of information as quickly as possible into field programs and into surveillance and control efforts. The path is certainly not an easy one, and previous premature celebrations over the apparent control of one or more of these diseases following the development or discovery of a new drug or pesticide, give pause to thoughts of success now. The vectors and pathogens are resourceful and unrelenting, and it is unlikely that any magic bullet will alleviate the situation. Multiple targets and approaches will undoubted be required in integrated management systems if we are to control any of these diseases.

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Pathogenesis of Rift Valley fever virus in mosquitoes – tracheal conduits & the basal lamina as an extra-cellular barrier

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Summary. Knowledge of the fate of an arbovirus in a mosquito is fundamental to understanding the mosquito's competence to transmit the virus. When a competent mosquito ingests viremic vertebrate blood, virus infects midgut epithelial cells and replicates, then disseminates to other tissues, including salivary glands and/or ovaries. The virus is then transmitted to the next vertebrate host horizontally via bite and/or vertically to the mosquito's offspring. Not all mosquitoes that ingest virus become infected or, if infected, transmit virus. Several "barriers" to arbovirus passage, and ultimately transmission, have been identified in incompetent or partially competent mosquitoes, including, among others, gut escape barriers and salivary gland infection barriers. The extra-cellular basal lamina around the midgut epithelium and the basal lamina that surrounds the salivary glands may act as such barriers. Midgut basal lamina pore sizes are significantly smaller than arboviruses and ultrastructural evidence suggests that midgut tracheae and tracheoles may provide a means for viruses to circumvent this barrier. Further, immunocytochemical evidence indicates the existence of a salivary gland infection barrier in *Anopheles stephensi*. The basal lamina may prevent access to mosquito cell surface virus receptors and help explain why anopheline mosquitoes are relatively incompetent arbovirus transmitters when compared to culicines.

Introduction

Arboviruses ("arthropod-borne viruses") are transmitted to vertebrates by mosquitoes and related diptera, and acarines (mites & ticks) and replicate in both their vertebrate and arthropod hosts. Mosquitoes are particularly important vectors of

these viruses [8, 9, 13, 23, 28]. Despite their serious impact on human and animal health, many of the interactions between arboviruses and their arthropod vectors remain poorly understood, especially at detailed histological, ultrastructural, and molecular levels.

When a fully competent mosquito ingests viremic vertebrate blood, virus infects midgut epithelial cells, replicates within these cells, disseminates from the midgut and infects tissues in the hemocoel, including the salivary glands and/or ovaries. The virus is then transmitted to the next vertebrate host via the salivary glands and/or to the mosquito's offspring via the ovaries. Not all mosquitoes that ingest virus become infected or, if infected, transmit virus. Many studies have revealed significant variation, among different species and various strains of the same species, both in susceptibility to oral infection and in the ability to transmit once infected [9]. Several "barriers", that delay or prevent arbovirus passage, have been identified at various junctures between ingestion and transmission to a new host. Some mosquitoes fail to develop a gut infection (gut infection "barrier"), or if the gut is infected, virus may fail to pass into the hemocoel or is slow in doing so (gut escape or dissemination "barrier"; e.g., [37]). In mosquitoes with disseminated infections, that is with virus in the hemocoel, the salivary glands may fail to become infected (salivary gland infection "barrier;" e.g. new information herein presented) or if infected the virus may not be transmitted (salivary gland escape "barrier"). These various "barriers" have been characterized as time-dependent, dose-dependent, and absolute.

Hypothetical mechanisms involved in "barriers" against virus passage can be divided into three groups: (1) those that operate extra-cellularly, e.g. the non-cellular basal lamina that may block access to cell surface receptors; (2) those that operate in association with the cell membrane, that is are related to the presence, absence, or density of mosquito cell surface virus receptor molecules; and (3) those that operate within mosquito cells and which cause a delay or failure in viral replication or exiting from infected host cells.

This paper deals specifically with the midgut basal lamina as an "extra-cellular barrier" mechanism, midgut tracheae as possible "conduits" through this barrier, and with the existence of a salivary gland infection barrier that is thought to involve the basal lamina.

In a discussion of western equine encephalitis virus in the midgut of the mosquito *Ochlerotatus dorsalis*, Hardy [9] points out: "... there is little direct evidence to explain how the virus is transported through the basal lamina of the mesenteron (midgut) into the hemocoel. The pore size in the basal lamina of *Ochlerotatus dorsalis* is only is only 10 nm in diameter ... the mechanism by which virions with a diameter of 50 to 60 nm can traverse the basal lamina of the mosquito mesenteron remains an enigma. The fact that they can go through this structure is suggested by the infection of the mesenteron in parenterally infected females." Romoser et al. [25] present evidence for the action of the midgut basal lamina as a barrier to arbovirus passage and for the concomitant operation of tissue "conduits", which may involve midgut tracheae and/or modified "spongy" basal lamina associated with midgut muscle.

Rift Valley fever virus (RVFV) (genus *Phlebovirus*, family *Bunyaviridae*) causes an emerging viral disease with high morbidity rates in humans and high mortality rates in domestic animals [20, 21]. The virion is spherical, approximately 100 nm in diameter, and has a negative sense, tripartite, RNA genome. A lipid envelope from which emerge several glycoprotein spikes makes up the outer layer of the virion. A mosquito, *Culex pipiens*, was implicated as primary vector in a major RVF outbreak in Egypt [19].

Anopheles albimanus Wiedemann is not a competent vector of RVFV and typically does not transmit this virus when infected by injection of virus directly into the hemocoel (IT or intrathoracic inoculation). However, if larvae or "young" pupae are IT-inoculated with virus, the adults are able to transmit it [28]. This indicates that in larvae or "young" pupae, virus enters cells in the primordial tissue which gives rise to the adult mosquito salivary glands, and that these glands become refractory to infection sometime later during metamorphosis. The same phenomenon occurs in *An. stephensi* Liston [30]. Based on the work of Turell [28] and Turell & Romoser [30], we have hypothesized the existence of a salivary gland infection barrier in adult female mosquitoes. Given its location between the hemocoel and the salivary gland epithelial cells, the basal lamina is an excellent candidate for the role of this barrier.

The objectives of this paper are as follows: (1) to present new information regarding the relationship between midgut tracheae, the basal lamina, midgut epithelial cells, and RVFV in *Cx. pipiens*; and (2) to present evidence of a salivary gland infection barrier to RVFV in *An. stephensi* that may be associated with the basal lamina.

Materials and methods

For ultrastructural studies, we used the mosquito *Cx. pipiens* (El Gabal strain). Mosquito rearing and all work involving infectious material was done at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) at Fort Detrick, Maryland while all preparation of ultrathin sections, histological sections and immunocytochemistry was done at Ohio University.

For histological analysis of *Cx. pipiens*, we used serial paraffin sections that had been prepared for previous immunocytochemical studies and which were stained with hemotoxylin [24]. We examined electron photomicrographs produced for earlier studies of RVFV (ZH501) in the mosquito *Cx. pipiens*, El Gabal strain [15]. In these earlier studies, viral plaque assay on Vero cells [7] was used to determine the initial "dose" of RVFV or Venezuelan encephalitis virus injected into mosquitoes, or introduced in a blood meal, and to determine the dissemination status of orally fed mosquitoes based on the presence of infectious particles in dissected legs.

We tested the hypothesis that there is a salivary gland infection barrier [30] in adult female *An. stephensi* mosquitoes. In order to produce a series of mosquitoes infected at different developmental stages, samples of fourth instar larvae, pupae of differing ages post-pupation, and adults (Table 1) were IT-inoculated with the ZH-501 strain of RVFV. In addition, uninfected, adult mosquitoes were IT-inoculated with diluent, prepared in the same way as infected mosquitoes, and used as negative controls in the immunocytochemical procedure. All specimens were incubated for equilivalent lengths of time (approximately 7 days following

Table 1. Rift Valley fever virus antigen and transmission status in adult *Anopheles stephensi* as a function of developmental stage at the time of inoculation, % (number antigen positive/total)

Developmental stage	Material injected	Non-salivary gland tissue	Salivary gland	Transmitted virus to hamster
Larve & Pupae <4 h (n = 20)	RVFV	100 (20/20)	95 (19/20)	72.2 (13/18)
Pupae >24 h (n = 7)	RVFV	100 (7/7)	57.1 (4/7)	16.7 (1/6)
Adult (n = 20)	RVFV	100 (20/20)	10 (2/20) 30 (6/20)[1]	0 (0/18)
Adult (n = 10)	Diluent	0 (0/10)	0 (0/10)	0 (0/10)

[1] ± Considered positive

inoculation), time enough for all specimens to reach the adult stage and become ready to blood feed. Most mosquitoes were tested for their ability to transmit virus to a Golden Syrian hamster. Specimens were then fixed in 10% formaldehyde for approximately 5 hours and stored in 70% ethyl alcohol. Serial paraffin sections were prepared according to standard microtechnical methods. To detect the presence of RVFV infection in mosquito tissues, we used the avidin-biotin-peroxidase complex (ABC) immunocytochemical technique [6, 38].

Results

Tracheal conduits and the midgut basal lamina as a barrier

It is well known that the mosquito midgut is tracheated. From ultrastructural examination, it is also clear that the proventriculus [15] is likewise tracheated and that the tracheal branches are intimately associated with the muscles of this structure (Figs. 1, 2).

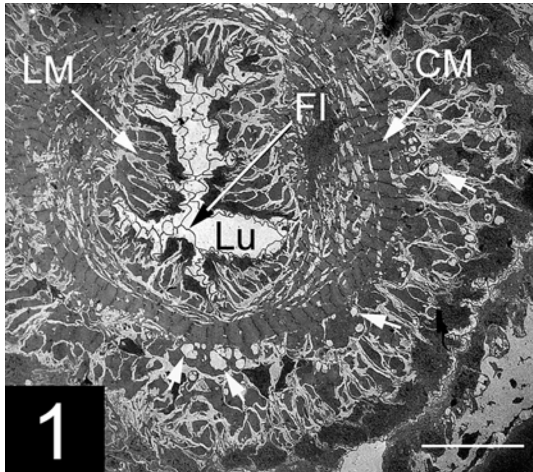

Fig. 1. Electron micrograph of cross-section of proventriculus of *Cx. pipiens*. *CM* Circular muscle; *FI* foregut intima; *LM* longitudinal muscle; *Lu* lumen; unlabeled white arrows, tracheae/tracheoles closely associated with muscle tissue (Scale bar: 8μ)

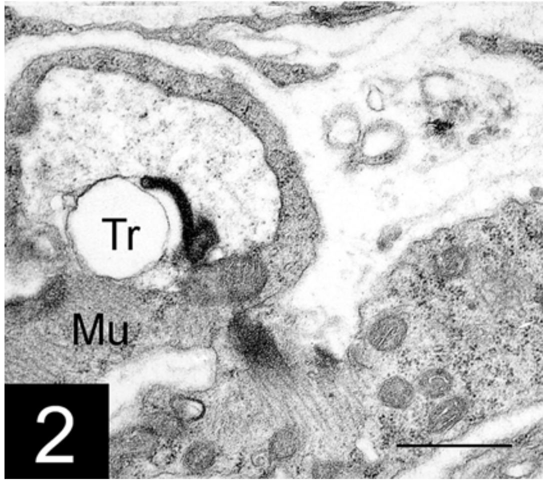

Fig. 2. Electron micrograph of section of a tracheo-muscular complex in the proventriculus of *Cx. pipiens*; *Mu* muscle; *Tr* tracheole (Scale bar: 2μ)

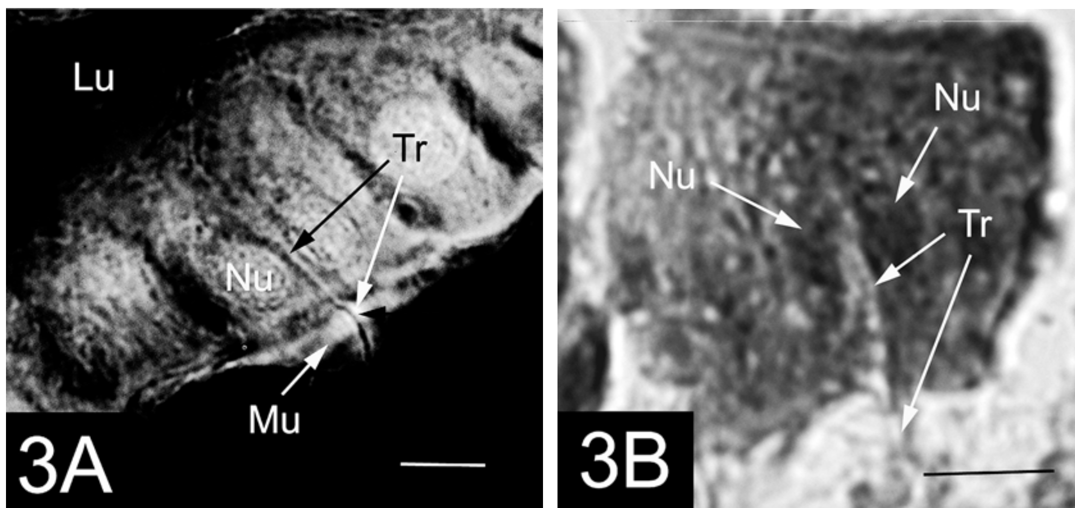

Fig. 3. (**A**, **B**) Histological sections of posterior midgut of *Cx. pipiens* showing apparent tracheal penetration of midgut epithelium. *Lu* Lumen of midgut; *Mu* muscle; *Nu* nucleus of midgut epithelial cell; *Tr* trachea (Scale bar: A, 10μ; B, 10μ)

Histological examination of several specimens of *Cx. pipiens* revealed that tracheae/tracheoles associated with muscles that surround the posterior midgut appear to penetrate the midgut epithelium at points along the midgut (Fig. 3). Figure 4 shows the presence of RVFV particles in the cytoplasm of a tracheole within the spongy basal lamina of the proventriculus in a specimen with a non-disseminated infection, as indicated by viral titration of dissected legs. Figure 5 shows the presence of putative RVFV particles in the "endocuticle" of the foregut intima and Fig. 6, the presence of putative RVFV particles in the "endocuticle" of a trachea.

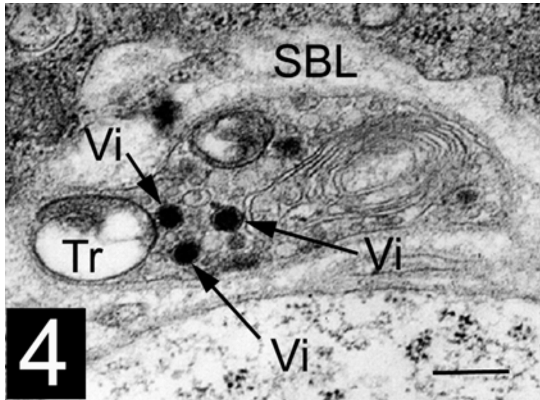

Fig. 4. Electron micrograph of tracheole within the proventriculus of *Cx. pipiens* from a mosquito with a non-disseminated Rift Valley fever virus infection. *Vi* Virion; *SBL* spongy basal lamina; *Tr* tracheole. (Scale Bar: 0.25μ)

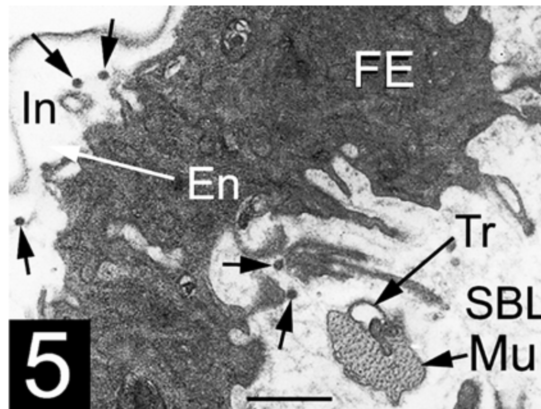

Fig. 5. Electron micrograph of intussuscepted foregut epithelium in proventriculus of *Cx. pipiens* showing Rift Valley fever virions (unlabeled black arrows) in the "endocuticle" of the foregut intima and in the spongy basal lamina. *En* Endocuticle of foregut intima; *FE* foregut epithelium; *In* intima (includes the "endocuticle" and the thin, darkly staining "epicuticle"); *Mu* muscle; *SBL* spongy basal lamina; *Tr* tracheole (Scale Bar: 1μ)

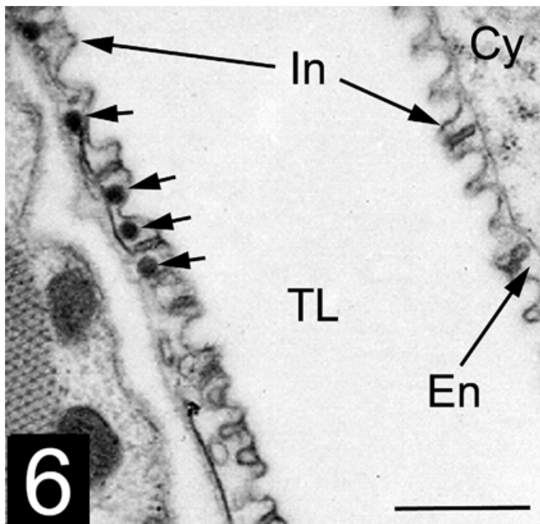

Fig. 6. Electron micrograph of trachea in the hemocoel of *Cx. pipiens* showing Rift Valley fever virions (arrowheads) within the "endocuticle" of the tracheal intima. *Cy* Cytoplasm of tracheal cell body; *En* endocuticle; *In* tracheal intima (includes the "endocuticle" and the thin, darkly staining "epicuticle"); *TL* lumen of trachea (Scale Bar: 0.5μ)

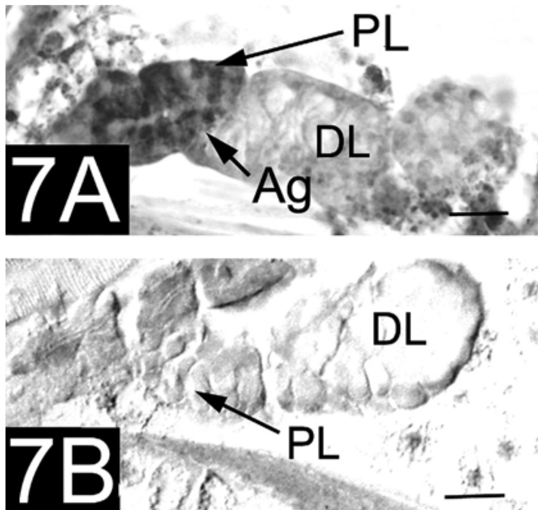

Fig. 7. Histological sections of *An. stephensi* salivary gland. (**A**) Rift Valley fever antigen-positive region in the proximal lobe of the salivary gland. (**B**) negative control. Ag, RVF viral antigen (the entire darkly staining region); *DL* distal lateral lobe of salivary gland; *PL* proximal lateral lobe of salivary gland (Scale Bar: 25µ)

The basal lamina as a possible salivary gland barrier

The results of our examination of the immunocytochemically stained slides are shown in Table 1. It is clear that although RVFV infection occurred in all the mosquitoes inoculated, the extent of salivary gland infection diminished as a function of developmental stage at the time of inoculation, being as low as 10% in mosquitoes inoculated as adults. Likewise, the percent transmission decreased as a function of developmental stage and no mosquitoes inoculated as adults transmitted virus.

Before being fixed and prepared for immunocytochemical study, most mosquitoes from among those inoculated as larvae or as adults, as well as those inoculated with diluent, were tested for their ability to transmit virus to hamsters. The identity of two of these mosquitoes that transmitted virus and two that did not was revealed to the investigators who analyzed the immunocytochemically stained slides. Four additional mosquitoes, which transmitted virus to hamsters and five additional mosquitoes that did not transmit, remained unknown to them. Using the "knowns" as guides, "unknowns" were examined to see if we could separate them into transmitters and non-transmitters on the basis of whether or not the salivary glands contained RVFV antigen and were therefore infected. We were able to do so with 100% accuracy. Figure 7 shows an RVFV antigen-positive salivary gland in a mosquito infected during the larval or early pupal stage (Fig. 7A) compared with an antigen-negative salivary gland in an uninfected adult mosquito (Fig. 7B).

Discussion

The basal lamina as a midgut barrier and tracheal conduits

Based on our results: (1) tracheae can be infected by RVFV; (2) tracheae/tracheoles appear to penetrate the basal lamina and hence are potentially in direct contact with infected midgut epithelial cells; (3) virions can occur in trachea on the hemocoel

side of the midgut in mosquitoes that show no evidence of dissemination on the basis of viral plaque assay of dissected legs. These results, viewed collectively, provide further support for the hypothesis that tracheae can serve as conduits for virus passage from the mosquito midgut into the hemocoel [25].

It is interesting to note the presence of RVFV particles in endocuticular portions of the foregut intima (Fig. 6) and tracheal endocuticle (Fig. 7). It is speculation, but we wonder whether, because virus is able to penetrate these cuticular zones, it is also able to move freely within them. If so the endocuticle of the tracheal system could possibly provide a fast track for virus movement throughout the mosquito without the necessity of serial episodes of tissue infection and replication before reaching the salivary glands, ovaries, or other tissues. Because tracheae end in cells (tracheoblasts) that are intimately associated with various organs, e.g. salivary glands and ovaries, virus conceivably could reach the tracheated organs via the endocuticle and infect these organs at the tracheoblast level. Viral dissemination via the tracheal endocuticle could account for the occasional early/rapid dissemination observed in mosquitoes infected with RVFV as well as other viruses [6, 9]. Bowers et al. [2] demonstrated the presence and persistence of Sindbis virus in tracheael cells of infected mosquitoes and suggested the possibility of viral spread to secondary tissue targets via the tracheal system and that, perhaps, viruses gain protection from antiviral mechanisms in the hemolymph by remaining within the tracheae. It is intriguing to note that Sindbis virus particles can be seen with the "endocuticle" of a gut-associated tracheole in one of their figures.

Baculoviruses appear to utilize a tracheal route of spread within their insect hosts [1, 5, 12]. Further, the earliest evidence of nuclear polyhedrosis infection beyond the midgut of larval *Trichoplusia ni* was in tracheoblasts and tracheal epidermal cells associated with foci of infection in the midgut epithelium [5]. They suggested that rapid spread of virus in the hemocoel could occur via intercellular spaces associated with tracheal epithelia.

Evidence of ovarian infection with LaCrosse virus before dissemination of the virus from the midgut suggested a possible tracheal route to the ovaries [4]. Thompson et al. [27] found that the dissemination rate of dengue viruses in *Aedes albopictus* did not vary with the thickness of the basal lamina, as one would expect if virus passed directly through this layer. Their results are therefore consistent with the basal lamina as a barrier and virus passage from the midgut via another route.

Complete tracheal penetration of the basal lamina putting tracheal cells in direct plasma membrane contact with midgut epithelial cells has been described in other kinds of insects [18, 35]. However, earlier studies of mosquito midgut ultrastructure did not describe tracheal penetration of the basal lamina [10, 11]. More study is needed to resolve the issue of tracheal penetration of the midgut epithelium in mosquitoes as well as the role of tracheae as conduits for virus passage.

The basal lamina as a possible salivary gland barrier

On the basis of the work of Turell [28] and Turell & Romoser [30], we have tested the hypothesis that there is a salivary gland infection barrier in adult female

An. stephensi mosquitoes. Our results at this point are somewhat preliminary, but are consistent with the basal lamina as an extracellular barrier to arbovirus passage.

Because only a single mosquito infected as an old pupa and none of those infected as adults transmitted virus, and comparatively few had detectable antigen in the salivary glands, it appears that there is an effective salivary gland infection barrier operating. Because transmission decreases as a function of developmental stage, it appears that the infection barrier develops some time during metamorphosis. That the salivary glands become infected during their development indicates that these cells are capable of supporting RVFV replication. The fact that a few mosquitoes inoculated as adults had RVFV antigen in the salivary glands shows that the cells which make up these organs express in the adult stage the gene(s) associated with the cell surface receptors for RVFV. Because the basal lamina would be deposited in temporal proximity with appearance of new adult salivary gland epithelium, it is an obvious candidate as a barrier. One might argue that virus may replicate at different rates depending on the timing of infection relative to developmental stage. However, Turell [28] found that when incubation times were equivalent, similar viral titers were produced regardless of the timing of infection.

Leake [13] points out "It is striking that anopheline mosquitoes have been only rarely associated with arboviruses. . .while culicine mosquitoes, principally members of the *Aedes* and *Culex* genera, predominate as virus vectors." The salivary gland infection barrier we have found in *An. stephensi* is consistent with anopheline incompetence to transmit arboviruses, and provides a possible explanation of these differences. That is the basal lamina in *Anopheles* mosquitoes may prevent contact between RVFV particles and their mosquito cell surface receptors, while failing to do so in *Aedes* and *Culex*.

Several studies have shown that co-infections of mosquitoes with a given arbovirus and a protozoan or metazoan parasite enhance aspects of the pathogenesis of arboviral infection. For example, infection (achieved by allowing mosquitoes to feed on co-infected vertebrate hosts) of a mosquito with eastern equine encephalitis virus or Venezuelan equine encephalitis virus, dengue 2 virus, or RVFV and co-infected, by ingestion, with *Brugia malayi* microfilariae enhanced the infectivity, dissemination, and transmission, respectively, of the associated arbovirus in *Aedes* and *Ochlerotatus* mosquitoes [29, 31, 33, 34]. Likewise, the ability of *An. stephensi* to transmit RVFV was enhanced by the presence of *Plasmodium berghei* sporozoites [33]. Both cases of enhanced arbovirus passage out of or into an epithelium encased in a layer of basal lamina support the role of the basal lamina as a barrier that must be penetrated in order for the arboviruses we studied to spread within a mosquito. That is, the penetration of the basal lamina of the midgut and/or salivary glands by the parasites facilitates arboviral passage that may otherwise depend upon tracheal, muscular, or other conduits, or passage that might not happen at all.

In conclusion, our results are consistent with the idea of the basal lamina as an extracellular barrier to arbovirus passage from the midgut into the hemocoel

and into the salivary glands in adult anopheline mosquitoes. The apparent involvement of the basal lamina in the dynamics of arboviral pathogenesis, which is supported by our work as well as by reports in the literature, makes understanding of this layer and with the conduits that enable its penetration by arboviruses, of crucial importance to our ultimate understanding of the transmission of arboviruses.

Acknowledgments

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The virulence of the 1918 pandemic influenza virus: unraveling the enigma

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Summary. The 1918 influenza pandemic caused acute illness in 25–30% of the world's population and resulted in the death of up to 40 million people. Using lung tissue of 1918 influenza victims, the complete genomic sequence of the 1918 influenza virus is being deduced. Neither the 1918 hemagglutinin nor neuraminidase genes possess mutations known to increase tissue tropicity that account for virulence of other influenza virus strains, such as A/WSN/33 or the highly pathogenic avian influenza H5 or H7 viruses. Using reverse genetics approaches, influenza virus constructs containing the 1918 hemagglutinin and neuraminidase on an A/WSN/33 virus background were lethal in mice. The genotypic basis of this virulence has not yet been elucidated. The complete sequence of the non-structural (NS) gene segment of the 1918 virus was deduced and also tested to determine the validity of the hypothesis that enhanced virulence in 1918 could have been due to type I interferon inhibition by the NS1 protein. Results from these experiments suggest that in human cells the 1918 NS1 is a very effective interferon antagonist. Sequence analysis of the 1918 influenza virus is allowing us to test hypotheses as to the origin and virulence of this strain. This information should help elucidate how pandemic influenza virus strains emerge and what genetic features contribute to virulence in humans.

Introduction

Influenza A viruses are negative strand RNA viruses of the family *Orthomyxoviridae*, genus *Influenzavirus A*. They continually circulate in humans in yearly epidemics (mainly in the winter in temperate climates) and antigenically novel virus strains emerge sporadically as pandemic viruses [11]. In the United States, influenza is estimated to kill 30,000 people in an average year [68, 75]. Every few years, influenza epidemics boost the annual number of deaths past the average, causing 10–15,000 additional deaths. Occasionally, and unpredictably, influenza sweeps the world, infecting 20% to 40% of the population in a single year. In these pandemic years, the numbers of deaths can be dramatically above average.

In 1957–1958, a pandemic was estimated to cause 66,000 excess deaths in the United States [67]. In 1918, the worst pandemic in recorded history was associated with approximately 675,000 total deaths in the United States [78], and killed an estimated 40 million people worldwide [12, 27, 52].

Studying the extent to which the 1918 influenza epidemic was like other pandemics may help us to understand how pandemic influenzas emerge and cause disease in general. On the other hand, if we determine what made the 1918 influenza pandemic different from other pandemics, we may use the lessons of the virulence of the 1918 influenza virus to predict the magnitude of public health risks a new pandemic virus might pose.

Historical background

The influenza pandemic of 1918 was exceptional in both breadth and depth. Outbreaks of the disease swept not only North America and Europe but also spread as far as the Alaskan wilderness and the most remote islands of the Pacific. It has been estimated that one-third of the world's population (500 million people) may have become infected and ill during the pandemic [6, 16]. The disease was also exceptionally severe, with mortality rates among the infected of more than 2.5%, compared to less than 0.1% in other influenza epidemics [44, 60]. Total mortality attributable to the 1918 pandemic was probably around 40 million [12, 27, 52].

Unlike most subsequent influenza virus strains that have developed in Asia, the 'first wave' or 'spring wave' of the 1918 pandemic seemingly arose in the United States in March, 1918 [1, 12, 28]. However, the near simultaneous appearance of influenza in March–April, 1918 in North America, Europe, and Asia makes definitive assignment of a geographic point of origin difficult [28]. It is possible that a mutation or reassortment occurred in the late summer of 1918, resulting in significantly enhanced virulence. The main wave of the global pandemic, the 'fall wave' or 'second wave,' occurred in September–November, 1918. In many places, there was yet another severe wave of influenza in early 1919 [28].

Three extensive outbreaks of influenza within one year is unusual, and may point to unique features of the 1918 virus that could be revealed in its sequence. Interpandemic influenza outbreaks generally occur in a single annual wave in the late winter. The severity of annual outbreaks is affected by antigenic drift, with an antigenic variant virus strain emerging every two to three years. Even in pandemic influenza, when the normal late winter seasonality pattern may be violated, the successive occurrence of distinct waves within a year is unusual. The 1890 pandemic began in the late spring of 1889 and took several months to spread throughout the world, peaking in northern Europe and the United States late in 1889 or early 1890. The second wave peaked in spring 1891 (more than a year after the first wave) and the third wave in early 1892 [28]. As in 1918, subsequent waves seemed to produce more severe illness, so that the peak mortality rate was reached in the third wave of the pandemic. The three waves, however, were spread over more than three years, in contrast to less than one year in 1918. It is unclear

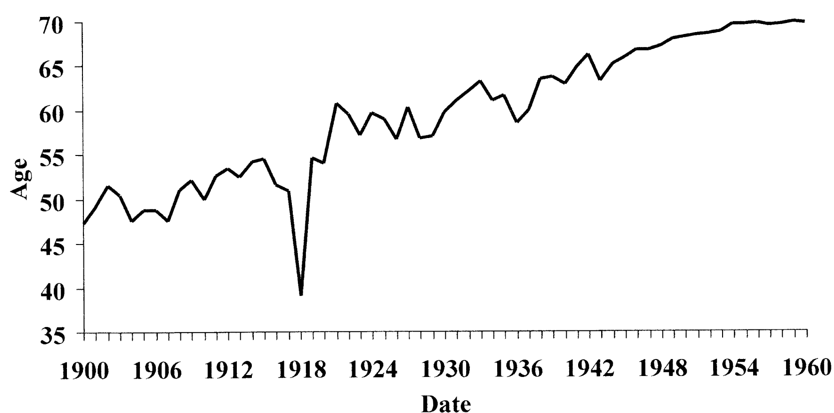

Fig. 1. Life expectancy in the United States, 1900–1960 showing the impact of the 1918 influenza pandemic [24, 42, 78]

what gave the 1918 virus this unusual ability to generate repeated waves of illness. Perhaps the surface proteins of the virus drifted more rapidly than they did in other influenza virus strains, or perhaps the virus had an unusually effective mechanism for evading the human immune system.

It has been estimated that the influenza epidemic of 1918 killed 675,000 Americans, including 43,000 servicemen mobilized for World War I [12]. The impact was so profound as to depress average life expectancy in the U.S. by more than 12 years, Fig. 1 [24], and may have played a significant role in ending the World War I conflict [12, 43].

The majority of individuals who died during the pandemic succumbed to secondary bacterial pneumonia [28, 39, 84], since no antibiotics were available in 1918. However, a subset died rapidly after the onset of symptoms, often with either massive acute pulmonary hemorrhage or pulmonary edema, and often in fewer than 5 days [39, 83, 84]. In the hundreds of autopsies performed in 1918, the primary pathologic findings were confined to the respiratory tree and death was due to pneumonia and respiratory failure [83]. These findings are consistent with infection by a well-adapted influenza virus capable of rapid replication throughout the entire respiratory tree [54, 72]. There was no clinical or pathological evidence for systemic circulation of the virus [83].

Furthermore, in the 1918 pandemic most deaths occurred among young adults, a group in which there usually is a very low death rate from influenza. Influenza and pneumonia death rates for 15–34 year olds were more than 20 times higher in 1918 than in previous years, Fig. 2 [42, 67]. The 1918 pandemic is also unique among influenza pandemics in that absolute risk of influenza mortality was higher in those less than 65 years of age than in those greater than 65. Strikingly, persons less than 65 years old accounted for greater than 99% of all excess influenza-related deaths in 1918–19 [67]. In contrast, the less-than-65 age group accounted for only 36% of all excess influenza-related mortality in the 1957 H2N2 pandemic and 48% in the 1968 H3N2 pandemic. Overall, nearly half of the influenza-related deaths in the 1918 influenza pandemic were young adults, age 20–40, Fig. 2 [67]. Why this

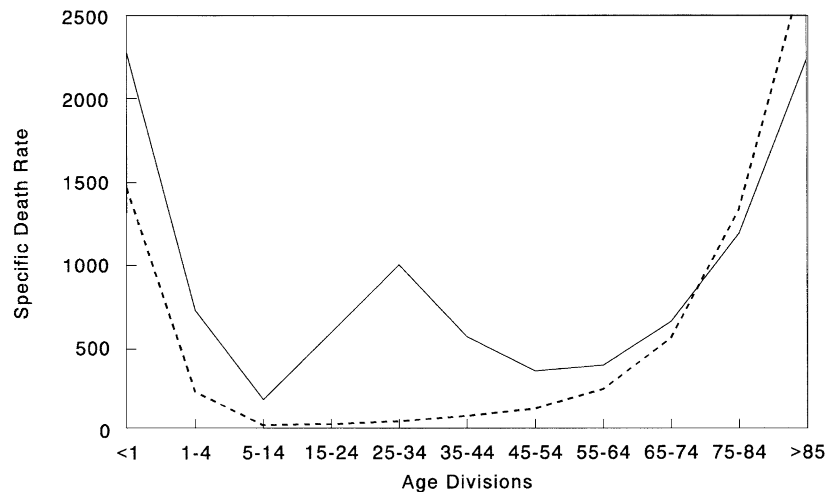

Fig. 2. Influenza and pneumonia mortality by age, United States. Influenza and pneumonia specific mortality by age, including an average of the inter-pandemic years 1911–1915 (dashed line), and the pandemic year 1918 (solid line). Specific death rate is per 100,000 of the population in each age division [24, 42, 78]

particular age group suffered such extreme mortality is not fully understood (see below).

The 1918 influenza had as another unique feature the simultaneous infection of both humans and swine. Interestingly, swine influenza was first recognized as a clinical entity in that species in the fall of 1918 [32] concurrent with the spread of the second wave of the pandemic in humans [14]. Investigators were impressed by clinical and pathological similarities of human and swine influenza in 1918 [32, 48]. An extensive review by the veterinarian W.W. Dimoch of the diseases of swine published in August 1918 makes no mention of any swine disease resembling influenza [13]. Thus, contemporary investigators were convinced that influenza virus had not circulated as an epizootic disease in swine before 1918 and that the virus spread from humans to pigs because of the appearance of illness in pigs after the first wave of the 1918 influenza in humans [66].

Thereafter the disease became widespread among swine herds in the U.S. midwest. The epizootic of 1919–1920 was as extensive as the one in 1918–1919. The disease then appeared among swine in the midwest every year, leading to Richard Shope's isolation of the first influenza virus in 1930, A/swine/Iowa/30 [65], three years before the isolation of the first human influenza virus, A/WS/33 by Smith, Andrewes, and Laidlaw [69]. Classical swine viruses have continued to circulate not only in North American pigs, but also in swine in Europe and Asia [4, 35, 49].

During the fall and winter of 1918–19, severe influenza-like outbreaks were noted not only in swine in the United States, but also in Europe and China [3, 9, 32]. Since 1918 there have been many examples of both H1N1 and H3N2 human influenza A virus strains becoming established in swine [5, 7, 86], while swine

influenza A virus strains have been isolated only sporadically from humans [21, 85].

The unusual severity of the 1918 pandemic and the exceptionally high mortality it caused among young adults have stimulated great interest in the influenza virus strain responsible for the 1918 outbreak [12, 33, 47]. Since the first human and swine influenza A viruses were not isolated until the early 1930's [65, 69], characterization of the 1918 virus strain has had previously to rely on indirect evidence [29, 64].

Epidemiological data on influenza prevalence by age in the population, collected between 1900 and 1918, provide good evidence for the emergence of an antigenically novel influenza virus in 1918 [28]. Jordan showed that from 1900–1917, the 5–15 age group accounted for 11% of total influenza cases in this series while the >65 age group similarly accounted for 6% of influenza cases. In 1918 the 5–15 year old group jumped to 25% of influenza cases, compatible with exposure to an antigenically novel virus strain but the >65 age group only accounted for 0.6% of the influenza cases in 1918. It is likely that the latter age group accounted for a significantly lower percentage of influenza cases because younger people were so susceptible to the novel virus strain (as seen in the 1957 pandemic [46, 67]) but it is also possible that these older people had pre-existing H1 antibodies. Further evidence for pre-existing H1 immunity can be derived from the age adjusted mortality data in Fig. 2. Those individuals >75 years had a lower influenza and pneumonia case mortality rate in 1918 than they had for the pre-pandemic period of 1911–1917.

When 1918 influenza case rates by age [28] are superimposed on the familiar 'W' shaped mortality curve (seen in Fig. 2), a different perspective emerges (Fig. 3). As shown, those <35 years of age in 1918 accounted for a disproportionately high influenza incidence by age. Interestingly, the 5–14 age group accounted for a large fraction of 1918 influenza cases, but had an extremely low case mortality rate compared to other age groups (Fig. 3). Why this age group had such a low case fatality rate cannot yet be fully explained. Conversely, why the 25–34 age group had such a high influenza and pneumonia mortality rate in 1918 remains enigmatic but it is one of the truly unique features of the 1918 influenza pandemic.

One theory that may explain these data concerns the possibility that the virus had an intrinsically high virulence that was tempered only in those patients who had been born before 1889. It can be speculated that the virus circulating prior to 1889 was an H1-like virus strain that provided partial protection against the 1918 virus strain [46, 67, 74].

Thus, it seems clear that the H1N1 virus of the 1918 pandemic contained an antigenically novel hemagglutinin and most humans and swine were susceptible to this virus in 1918. Given the severity of the pandemic, it is also reasonable to suggest that the other dominant surface protein, neuraminidase, would also have been replaced by antigenic shift before the start of the pandemic [54, 72]. In fact, sequence and phylogenetic analyses suggest that the genes encoding these two surface proteins were derived from an avian-like influenza virus shortly before the start of the 1918 pandemic and that the precursor virus did not circulate widely in

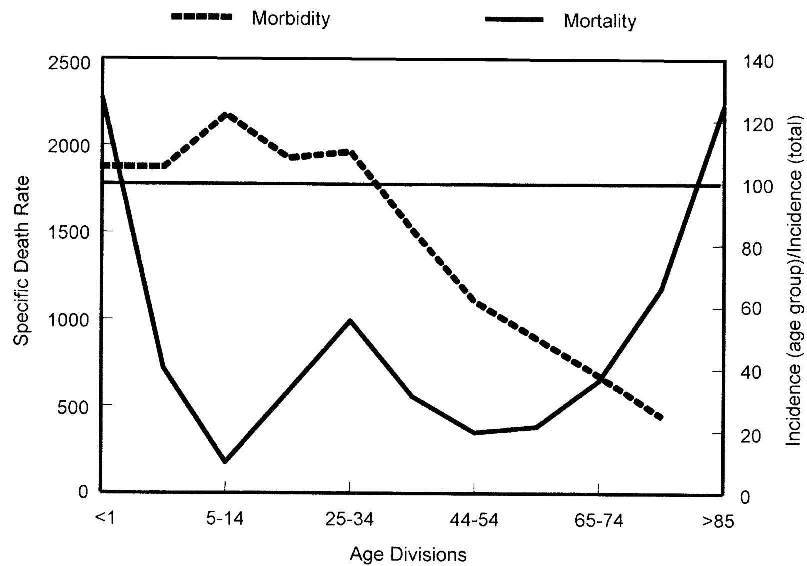

Fig. 3. Influenza and pneumonia mortality by age (solid line), with influenza morbidity by age (dashed line) superimposed. Influenza and pneumonia mortality by age as in Fig. 2. Specific death rate per age group, left ordinal axis. Influenza morbidity presented as ratio of incidence in persons of each group to incidence in persons of all ages (=100), right ordinal axis. Horizontal line at 100 (right ordinal axis) represents average influenza incidence in the total population [74] (Adapted from [28])

either humans or swine before 1918 [15, 53, 55]. It is currently unclear what other influenza gene segments were novel in the 1918 pandemic virus in comparison to the previously circulating virus strain. It is possible that on-going sequence and phylogenetic analyses of the gene segments of the 1918 virus may help elucidate this question.

Genetic characterization of the 1918 virus

Sequence and functional analysis of the hemagglutinin and neuraminidase gene segments

Frozen and fixed lung tissue from five fall wave 1918 influenza victims has been used to examine directly the genetic structure of the 1918 influenza virus. Two of the cases analyzed were U.S. Army soldiers who died in September, 1918, one in Camp Upton, New York and the other in Fort Jackson, South Carolina. The available material consists of formalin-fixed, paraffin-embedded (FFPE) autopsy tissue, hematoxylin and eosin-stained microscopic sections, and the clinical histories of these patients. A third sample was obtained from an Alaskan Inuit woman who had been interred in permafrost in Brevig Mission, Alaska, since her death from influenza in November 1918. The influenza virus sequences derived from these three cases have been called A/South Carolina/1/18 (H1N1), A/New York/1/18 (H1N1), and A/Brevig Mission/1/18 (H1N1), respectively. More recently, partial hemagglutinin (HA) sequence of two additional fixed autopsy

cases of 1918 influenza victims from the Royal London Hospital were determined [57]. The HA sequences from these five cases show $>99\%$ sequence identity, but differ at amino acid residue 225 (see below). To date, five 1918 influenza gene segment sequences have been published [2, 53, 55, 56, 59]. The sequences of the three polymerase genes are nearing completion.

The sequence of the 1918 HA is most closely related to that of the A/swine/Iowa/30 virus. However, despite this similarity the sequence has many avian-like structural features. Of the 41 amino acids that have been shown to be targets of the immune system and subject to antigenic drift pressure in humans, 37 match the avian sequence consensus, suggesting that there was little immunologic pressure on the HA protein before the fall of 1918 [53]. Another mechanism by which influenza viruses evade the human immune system is the acquisition of glycosylation sites to mask antigenic epitopes. The HAs from modern H1N1 viruses have up to five glycosylation sites in addition to the four found in all avian HAs. The HA of the 1918 virus has only the four conserved avian sites [53].

Influenza virus infection requires binding of the HA protein to sialic acid receptors on the host cell surface. The HA receptor binding site consists of a subset of amino acids that are invariant in all avian HAs but vary in mammalian-adapted HAs. Human-adapted influenza viruses preferentially bind sialic acid receptors with α(2–6) linkages. Those viral strains adapted to birds preferentially bind α(2–3) linked sugars [17, 45, 81]. To shift from the proposed avian-adapted receptor binding site configuration (with a preference for α(2–3) sialic acids) to that of swine H1s (which can bind both α(2–3) and α(2–6)) requires only one amino acid change, E190D. The HA sequences of all five 1918 cases have the E190D change [57]. In fact, the critical amino acids in the receptor-binding site of two of the 1918 cases are identical to that of the A/swine/Iowa/30 HA. The other three 1918 cases have an additional change from the avian consensus, G225D. Since swine viruses with the same receptor site as A/swine/Iowa/30 bind both avian and mammalian-type receptors [17], A/New York/1/18 virus probably also had the capacity to bind both. The change at residue 190 may represent the minimal change necessary to allow an avian H1-subtype HA to bind mammalian-type receptors [18, 23, 53, 57, 70], a critical step in host adaptation.

The crystal structure analysis of the 1918 HA [18, 70] suggests that the overall structure of the receptor binding site is akin to that of an avian H5 HA in terms of its having a narrower pocket than that identified for the human H3 HA [82]. This provides an additional clue for an avian-like derivation of the 1918 HA. The four antigenic sites that have been identified for another H1 HA, the A/PR/8/34 virus HA [8], also appear to be the major antigenic determinants on the 1918 HA. X-ray analyses suggest that these sites are exposed on the 1918 HA and thus they could be readily recognized by the human immune system.

The principal biological role of neuraminidase (NA) is the cleavage of the terminal sialic acid residues that are receptors for the virus' HA protein [51]. The active site of the enzyme consists of 15 invariant amino acids that are conserved in the 1918 NA. The functional NA protein is configured as a homotetramer in which the active sites are found on a terminal knob carried on a thin stalk [10].

Some early human virus strains have short (11–16 amino acids) deletions in the stalk region, as do many virus strains isolated from chickens. The 1918 NA has a full-length stalk and has only the glycosylation sites shared by avian N1 virus strains [62]. Although the antigenic sites on human-adapted N1 neuraminidases have not been definitively mapped, it is possible to align the N1 sequences with N2 subtype NAs and examine the N2 antigenic sites for evidence of drift in N1. There are 22 amino acids on the N2 protein that may function in antigenic epitopes [10]. The 1918 NA matches the avian consensus at 21 of these sites [55]. This finding suggests that the 1918 NA, like the 1918 HA, had not circulated long in humans before the pandemic and very possibly had an avian-like origin [58].

Neither the 1918 HA nor NA genes have obvious genetic features that can be related directly to virulence. Two known mutations that can dramatically affect the virulence of influenza virus strains have been described. For viral activation HA must be cleaved into two pieces, HA1 and HA2 by a host protease [38, 61]. Some avian H5 and H7 subtype viruses acquire a mutation that involves the addition of one or more basic amino acids to the cleavage site, allowing HA activation by ubiquitous proteases [31, 80]. Infection with such a pantropic virus strain can cause systemic disease with high mortality in birds. This mutation was not observed in the 1918 virus [53, 73].

The second mutation with a significant effect on virulence through pantropism has been identified in the NA gene of two mouse-adapted influenza virus strains, A/WSN/33 and A/NWS/33. Mutations at a single codon (N146R or N146Y, leading to the loss of a glycosylation site) appear, like the HA cleavage site mutation, to allow the virus to replicate in many tissues outside the respiratory tract [40]. This mutation was also not observed in the NA of the 1918 virus [55].

Therefore, neither surface protein-encoding gene has known mutations that would allow the 1918 virus to become pantropic. Since clinical and pathological findings in 1918 showed no evidence of replication outside the respiratory system [83, 84], mutations allowing the 1918 virus to replicate systemically would not have been expected. However, the relationship of other structural features of these proteins (aside from their presumed antigenic novelty) to virulence remains unknown. In their overall structural and functional characteristics, the 1918 HA and NA are avian-like but they also have mammalian-adapted characteristics.

Interestingly, recombinant influenza viruses containing the 1918 HA and NA and up to three additional genes derived from the 1918 virus (the other genes being derived from the A/WSN/33 virus) were all highly virulent in mice [77]. Furthermore, expression microarray analysis performed on whole lung tissue of mice infected with the 1918 HA/NA recombinant showed increased upregulation of genes involved in apoptosis, tissue injury and oxidative damage [30]. These findings were unusual because the viruses with the 1918 genes had not been adapted to mice. One explanation is that the combination of the genes/proteins of the 1918 virus was "optimal" because the 1918 genes possibly work synergistically in terms of virulence. The completion of the sequence of the entire genome of

the 1918 virus and the reconstruction and characterization of viruses with 1918 genes under appropriate biocontainment conditions will shed more light on this hypothesis and should allow a definitive examination of this explanation.

Antigenic analysis of recombinant viruses possessing the 1918 HA and NA by hemagglutination inhibition tests using ferret and chicken antisera suggested a close relationship with the A/swine/Iowa/30 virus and H1N1 viruses isolated in the 1930s [77], further supporting data of Shope from the 1930's [66]. Interestingly, when mice were immunized with different H1N1 virus strains, challenge studies using the 1918-like viruses revealed partial protection by this treatment suggesting that current vaccination strategies are adequate against a 1918-like virus [77]. In fact, the data may even allow us to suggest that the human population, having experienced a long period of exposure to H1N1 viruses, may be partially protected against a 1918-like virus [77].

Sequence and functional analysis of the non-structural gene segment

The complete coding sequence of the 1918 non-structural (NS) segment was completed [2]. The functions of the two proteins, NS1 and NS2 (NEP), encoded by overlapping reading frames [37] of the NS segment are still being elucidated [19, 20, 34, 41, 50]. The NS1 protein has been shown to prevent type I interferon (IFN) production, by preventing activation of the latent transcription factors IRF-3 [71] and NF-κB [79]. One of the distinctive clinical characteristics of the 1918 influenza was its ability to produce rapid and extensive damage to both the upper and lower respiratory epithelium [83]. Such a clinical course suggests a virus that replicated to a high titer and spread quickly from cell to cell. Thus, an NS1 protein that was especially effective at blocking the type I IFN system might have contributed to the exceptional virulence of the 1918 virus strain [19, 71, 79]. To address this possibility, transfectant A/WSN/33 influenza viruses were constructed with the 1918 NS1 gene or with the entire 1918 NS segment (coding for both NS1 and NS2 (NEP) proteins) [2]. In both cases, viruses containing 1918 NS genes were attenuated in mice compared to wild-type A/WSN/33 controls. The attenuation demonstrates that NS1 is critical for the virulence of A/WSN/33 in mice and suggests that NS1-related interferon antagonism is host specific. This is supported by transcriptional profiling (microarray analysis) of infected human lung epithelial cells that showed that a virus with the 1918 NS1 gene was more effective at blocking the expression of IFN-regulated genes than the isogenic parental mouse-adapted A/WSN/33 virus [22] suggesting that the 1918 NS1 contributes virulence characteristics in human cells but not murine ones. The 1918 NS1 protein varies from that of the WSN virus at 10 amino acid positions. The amino acid differences between the 1918 and A/WSN/33 NS segments may be important in the adaptation of the latter virus strain to mice and likely account for the observed differences in virulence in these experiments. Recently, a single amino acid change (D92E) in the NS1 protein was associated with increased virulence of the 1997 Hong Kong H5N1 viruses in a swine model [63]. This amino acid change was not found in the 1918 NS1 protein.

Sequence and functional analysis of the matrix gene segment

The coding region of influenza A RNA segment 7 from the 1918 pandemic virus, consisting of the open reading frames of the two matrix genes, M1 and M2, has been sequenced [56]. While this segment is highly conserved among influenza virus strains, the 1918 sequence does not match any previously sequenced influenza virus strains. The 1918 sequence matches the consensus over the M1 RNA-binding domains and nuclear localization signal and the highly conserved transmembrane domain of M2. Amino acid changes that correlate with high yield and pathogenicity in animal models were not found in the 1918 virus strain.

The M1 mRNA is colinear with the viral RNA, while the M2 mRNA is encoded by a spliced transcript [36]. The proteins encoded by these mRNAs share their initial nine amino acids and also have a stretch of 14 amino acids in overlapping reading frames. The M1 protein is a highly conserved 252 amino acid protein. It is the most abundant protein in the viral particle, lining the inner layer of the viral membrane and contacting the ribonucleoprotein core. M1 has been shown to have several functions [36] including regulation of nuclear export of vRNPs, both permitting the transport of vRNP particles into the nucleus upon infection and preventing newly exported vRNP particles from re-entering the nucleus. The 97 amino acid M2 protein is a homotetrameric integral membrane protein that exhibits ion channel activity and is the target of the drug amantadine [25]. The ion channel activity of M2 is important both during virion uncoating and during viral budding [36].

Five amino acid sites have been identified in the transmembrane region of the M2 protein that are involved in resistance to the antiviral drug, amantadine: sites 26, 27, 30, 31 and 34 [26]. The 1918 influenza M2 sequence is identical at these positions to that of the amantadine sensitive influenza virus strains. Thus, it was predicted that the M2 protein of the 1918 influenza virus would be sensitive to amantadine. This was recently demonstrated experimentally. A recombinant virus possessing the 1918 matrix segment was inhibited effectively both in tissue culture and *in vivo* by the M2 ion-channel inhibitors amantadine and rimantadine [76].

Future work

Five of the eight RNA segments of the 1918 influenza virus have been sequenced and analyzed. Their characterization has shed light on the origin of the virus and strongly supports the hypothesis that the 1918 virus was the common ancestor of both subsequent human and swine H1N1 lineages. Sequence analysis of the genes to date offers no definitive clue as to the exceptional virulence of the 1918 virus strain. To address hypotheses of virulence, in experiments using reverse genetics approaches with 1918 influenza genes have begun.

In future work it is hoped that the 1918 pandemic virus strain can be placed in the context of influenza virus strains that preceded and followed it. The direct precursor of the pandemic virus, the first or 'spring' wave virus strain, lacked the exceptional virulence of the fall wave virus strain. Identification of an influenza RNA-positive case from the first wave would have tremendous value in

deciphering the genetic basis for virulence by allowing differences in the sequences to be highlighted. Identification of pre-1918 human influenza RNA samples would clarify which gene segments were novel in the 1918 virus.

In many respects, the 1918 influenza pandemic was similar to other influenza pandemics. In its epidemiology, disease course and pathology, the pandemic generally was different in degree but not in kind from previous and subsequent pandemics. Furthermore, laboratory experiments using recombinant influenza viruses containing genes from the 1918 virus suggest that the 1918 and 1918-like viruses would be as sensitive to the FDA-approved anti-influenza drugs rimantadine and oseltamivir as are other typical virus strains [76]. However, there are some characteristics of the pandemic that appear to be unique: Mortality was exceptionally high, ranging from five to twenty times higher than normal. Clinically and pathologically, the high mortality appears to be the result of a higher proportion of severe and complicated infections of the respiratory tract, not with systemic infection or involvement of organ systems outside the influenza virus' normal targets. The mortality was concentrated in an unusually young age group. Finally, the waves of influenza virus activity followed on each other unusually rapidly, resulting in three major outbreaks within a year's time. Each of these unique characteristics may find their explanation in genetic features of the 1918 virus. The challenge will be in determining the links between the biological capabilities of the virus and the known history of the pandemic.

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The spread of the H5N1 bird flu epidemic in Asia in 2004

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Summary. H5N1 avian influenza has spread to eight countries in eastern Asia including China, Japan, South Korea, Vietnam, Laos, Cambodia, Thailand, and Indonesia in early 2004. This H5N1 influenza A virus is extremely virulent in poultry including chickens and ducks, killing millions of birds throughout the region. Additionally this virus has transmitted to humans (mainly children) in Vietnam, Cambodia, and Thailand, killing 54 of 100 diagnosed persons. To control this epidemic hundreds of millions of chickens and ducks have been culled. One genotype of H5N1 designated "Z" has become dominant in Asia. This virus was first detected in wild birds in Hong Kong in November 2002 and was antigenically distinct from H5N1 viruses isolated from 1997 to early 2002 and lethal for aquatic birds.

The H5N1 virus infecting humans and poultry in Asia in 2004 is an antigenic variant of the Z genotype. Here we consider the possible role of migrating birds in the evolution and spread of the H5N1 influenza A virus throughout Asia. We conclude that the available information is consistent with a role for migrating birds but limited information is available and that serological studies are urgently needed on migrating birds worldwide. The prospect is that this H5N1/04 influenza A virus will become endemic in poultry in eastern Asia and will be a continuing threat to animal and human health. It is also projected that a human H5N1 vaccine will eventually be needed.

Introduction

Since 1997 H5N1 influenza A viruses (family *Orthomyxoviridae*, *genus Influenzavirus* A) have continued to evolve in Asia. By early 2004 H5N1 variants had spread to at least eight countries including South Korea, Japan, Vietnam, Thailand, Cambodia, Laos, Indonesia and China. The virus is highly pathogenic for domestic poultry, especially chickens, and has killed millions of birds. Additionally this H5N1 virus has transmitted to at least 100 persons killing 54 of them.

The extent of the highly pathogenic H5N1 avian influenza epidemic in poultry in Asia is unprecedented. Poultry, particularly chickens, have become the main

protein source for humans in Asia and the health of poultry and of this industry is severely threatened. In humans (mainly children) this virus causes severe respiratory disease, lymphopenia, lung abnormalities and diarrhea with a high risk of death [16]. One of the many unanswered questions about this H5N1 virus is how it spread so quickly over much of eastern Asia. In this report we will first consider the evolution of this virus, review the existing knowledge about influenza A virus in migrating birds, then attempt to answer the question as to whether migrating birds played a role in its spread.

Evolution of the 2004 H5N1 influenza A virus

In 1997 H5N1 first transmitted to humans in Hong Kong and killed 6 of 18 persons before being eradicated by slaughter of all poultry in Hong Kong [7, 36, 42, 6]. This virus was a reassortant that obtained its hemagglutinin gene from A/Goose/Guangdong/1/96 (H5N1) and its seven other genes from influenza A viruses in quail. Epidemiological studies established that live bird markets were the source of infection [25]. Slaughter of poultry stopped the spread of this virus in humans and this particular genotype has not been found since. However, the precursor A/Goose/Guangdong/1/96 (H5N1) virus continued to circulate in ducks and geese in southeast China [5, 46]. In 2000 reassortant A/Goose/Guangdong/1/96 (H5N1)-like viruses were detected in geese and ducks that contained the same hemagglutination (HA) and neuraminidase (NA) as the 1996 H5N1 virus but with "internal" genes from unknown avian sources in Asia [13].

In 2001 H5N1 viruses re-emerged in Hong Kong poultry markets – with the same HA and NA but with five different "internal" gene constellations [12] (Fig. 1). The poultry in Hong Kong were again slaughtered to remove these genotypes. The H5N1 viruses continued to reassort with unknown avian influenza A viruses and by early 2002 additional H5N1 genotypes re-emerged in Hong Kong poultry markets and on poultry farms in Hong Kong. The HA of these viruses was antigenically and genetically close to A/Goose/Guangdong/1/96 (H5N1). Thus from 1997 until early 2002 the H5N1 viruses in Southeast Asia reassorted promiscuously, generating a range of different genotypes but the HA and NA remained largely conserved and similar to A/Goose/Guangdong/1/96 (H5N1) (Fig. 1).

This changed dramatically in November 2002 when the H5N1 viruses showed marked novel evolutionary properties: (i) The viruses were first detected in dead wild migratory birds (egrets, herons) [9]; (ii) These H5N1/02 viruses showed marked antigenic drift in the HA [41]; (iii) These H5N1/02 viruses killed most of the aquatic birds in nature parks in Hong Kong. Experimentally, the virus was highly lethal in ducks [41]. Influenza A viruses, including highly pathogenic H5 and H7 avian influenza A viruses, rarely kill ducks, which are the natural reservoir of influenza A viruses [45]. Thus, the pathogenicity in aquatic birds was unusual and noteworthy; (iv) One of the multiple genotypes (Z) transmitted to humans and killed one of a family of four in February 2003 [32]. A second child in the family died of similar symptoms in Fujian, China, but the cause was not determined.

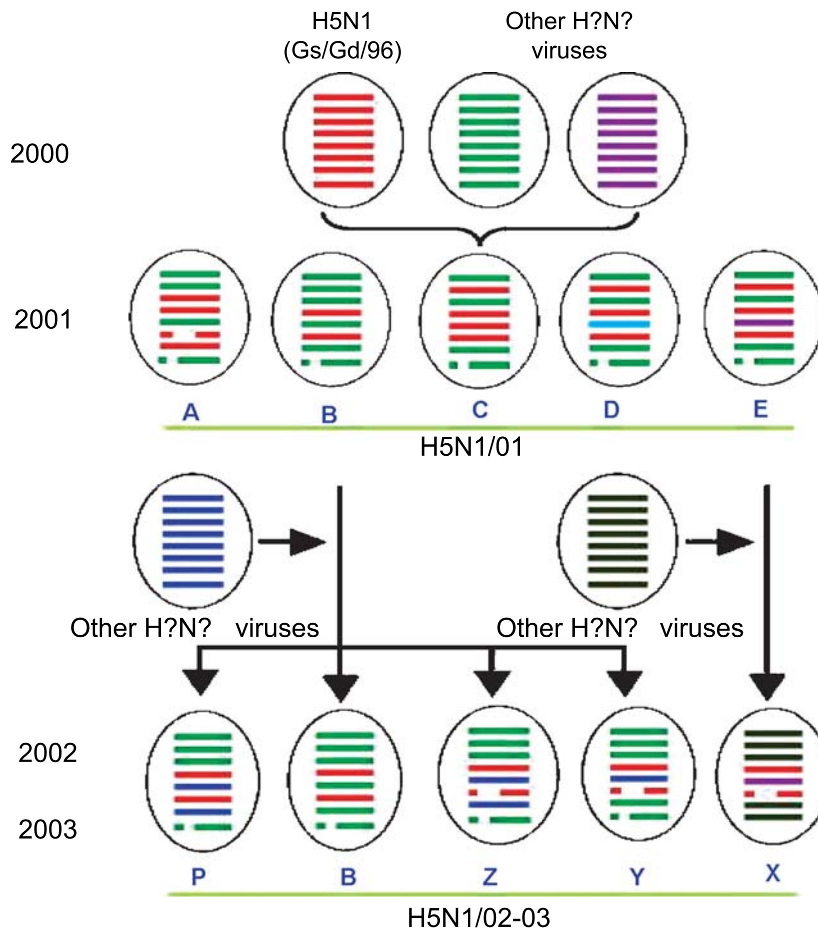

Fig. 1. The derivation of H5N1 reassortants in years 2000 through 2003. Reassortment is proposed between influenza A viruses to generate viruses with different gene constellations. The eight gene segments schematically shown in each virus particle encode (top to bottom) PB2, PB1, polymerase (*PA*), hemagglutinin (*HA*), nucleoprotein (*NP*), neuraminidase (*NA*), matrix (*M*), and nonstructural proteins (*NS*). Color coding denotes virus lineage. A gap in the NA or NS gene segment denotes a deletion

In early 2003 we predicted that this virus may have the potential to be spread by migrating birds and warned the World Health Organization (WHO) in February 2003 of this concern. By mid-2003, highly pathogenic H5N1 had emerged in poultry in Vietnam, Thailand and Indonesia but for political and economic reasons it was not reported until January 2004 when humans began to die in Vietnam and the "top blew off."

Influenza in migrating birds

There is general acceptance that the aquatic birds of the world are the natural reservoirs of influenza A viruses that infect other species including humans [45]. These viruses appear to be in evolutionary stasis while they are in wild aquatic

birds, where they cause asymptomatic infection. However, they evolve rapidly when they spread to other species and can cause mild to severe disease [37, 17, 35, 24, 18, 38, 43, 29]. Most of the available data come from studies on wild ducks; less data are available regarding influenza A viruses in other aquatic birds [20]. An unanswered question is "What are the reservoirs of the majority of the 15 H and 9 N subtypes that have been isolated periodically from apparently normal aquatic birds?"

The establishment of multiple lineages of H9N2 influenza A virus in domestic poultry in Eurasia [3, 11, 27, 23] and the transient transmission of H9N2 viruses to humans and pigs in southeast China [33, 31, 28] are examples that illustrate the role of aquatic birds in the evolution of influenza A viruses that transmit to mammals. Other examples include transmission of H5N1 influenza A virus in Asia through geese, ducks, and quail to humans in 1997 and again in 2003 and 2004 [7, 42, 47, 5, 15, 20, 12]. In North America, the emergence and re-emergence of H7N2 influenza A viruses in live poultry markets in the New York region [30]; the emergence of H7N7 in The Netherlands in 2003 [10], and the outbreak of H7N1 in Italy in 2000 [2] again illustrate the continuing role of the aquatic bird reservoir in the emergence and re-emergence of influenza A viruses of agricultural and human relevance.

Long term surveillance of influenza in migrating birds in North America

Surveillance for orthomyxoviruses in wild ducks over 26 years and in shorebirds over 16 years in North America established that influenza A viruses are perpetuated in both of these groups of wild aquatic birds [21]. Influenza B and C viruses were never isolated. Of the 15 known HA subtypes of influenza A virus, H1 through H12 were isolated from wild ducks and H1 through H13 from shorebirds. Subtype H13 was not isolated from wild ducks, and neither H14 nor H15 was isolated from either ducks or shorebirds in North America.

Comparison of the frequency of different HA subtypes of influenza A viruses isolated from wild ducks and shorebirds revealed that shorebirds carry a wider distribution of HA subtypes of influenza A viruses than do wild ducks. Thus, 9 of the 13 subtypes isolated in North America are isolated more frequently from shorebirds, whereas 3 of the 13 subtypes are isolated more frequently from wild ducks [21].

Although some investigators support the hypothesis that influenza A viruses are perpetuated in frozen lakes [28, 19], another possibility is that different families of aquatic birds are involved in perpetuating these orthomyxoviruses. There are at least 17 North American waterfowl that have western and eastern palearctic distribution [8]. With so many species having distributions throughout large portions of the northern hemisphere, it is difficult to imagine that mixing does not occur regularly. Waterfowl banding over many years, and more recently satellite telemetry, have shown that some birds marked in North America move to other continents for some portion of the year. Probably most of the mixing occurs

between Alaska and Northeastern Siberia, since they are so close to each other. Despite this mixing, phylogenetic lineages based on geographical separation are maintained by an undefined mechanism.

Of the influenza A viruses known to cause disease problems in humans and pigs, the H2 subtype is isolated more frequently from shorebirds, whereas subtypes H1 and H3 are found frequently in both wild ducks and shorebirds. The subtypes H5 and H7 that cause severe disease in domestic avian species were isolated more frequently from shorebirds than from ducks. This pattern is particularly applicable to H5, which is rarely isolated from wild ducks in North America. Other studies found H7 in wild ducks more frequently, but again H5 was rarely isolated [14]. Influenza A virus subtype H9, which causes less-severe disease in domestic avian species, has established permanent lineages in Eurasian poultry [3, 22] and more frequently occurs in shorebirds than ducks. In comparison, subtype H6, which is causing problems in poultry in the western U.S. [44], is more abundant in wild ducks than in shorebirds.

Because detailed surveillance has been done with aquatic birds of only a very few genera, the true reservoirs of the rarely isolated influenza A viruses probably remain to be found.

Migration routes and influenza A virus in wild aquatic birds in Asia

Wild aquatic birds in Asia likely are a crucial factor in the maintenance and zoonosis of influenza A viruses. In the Asia-Pacific region there are in excess of 400 species of aquatic birds and greater than 240 species of migratory birds. Migration occurs primarily in a north-south direction across three major flyways (Fig. 2), covers more than 50 countries, and extends from Siberia/western Alaska to Australia/New Zealand. The two flyways most affecting eastern Asia are the West Pacific flyway and the East Asian-Australasian flyway. Considerable overlap exists between flyways, particularly in the northern breeding grounds. Thus, there exists great potential for influenza A viruses, including highly pathogenic H5 and H7 strains, to be transported very long distances. The characteristic migration pattern of a particular flyway possibly will dictate the frequency and extent of spread of influenza A viruses across this region. For example, the West Pacific flyway is traversed by species that typically make extremely long migrations over open water, but the East Asian-Australasian flyway is characterized by "short-hops" along the coast. Hence, birds using the West Pacific flyway have less contact with land, and this could explain why New Zealand and the Philippines have not experienced outbreaks of highly pathogenic H5 or H7, while neighboring Australia has been the victim of several outbreaks. On the other hand, birds that traverse the East Asia-Australasian (coastal) route make land-fall frequently, and this pattern of migration might be responsible for the 2003–2004 outbreaks of highly pathogenic avian influenza A virus (HPAI) H5 in Vietnam, China, Cambodia, Thailand, and Laos. In this way some countries remain free from influenza infection while other countries in this region are severely affected.

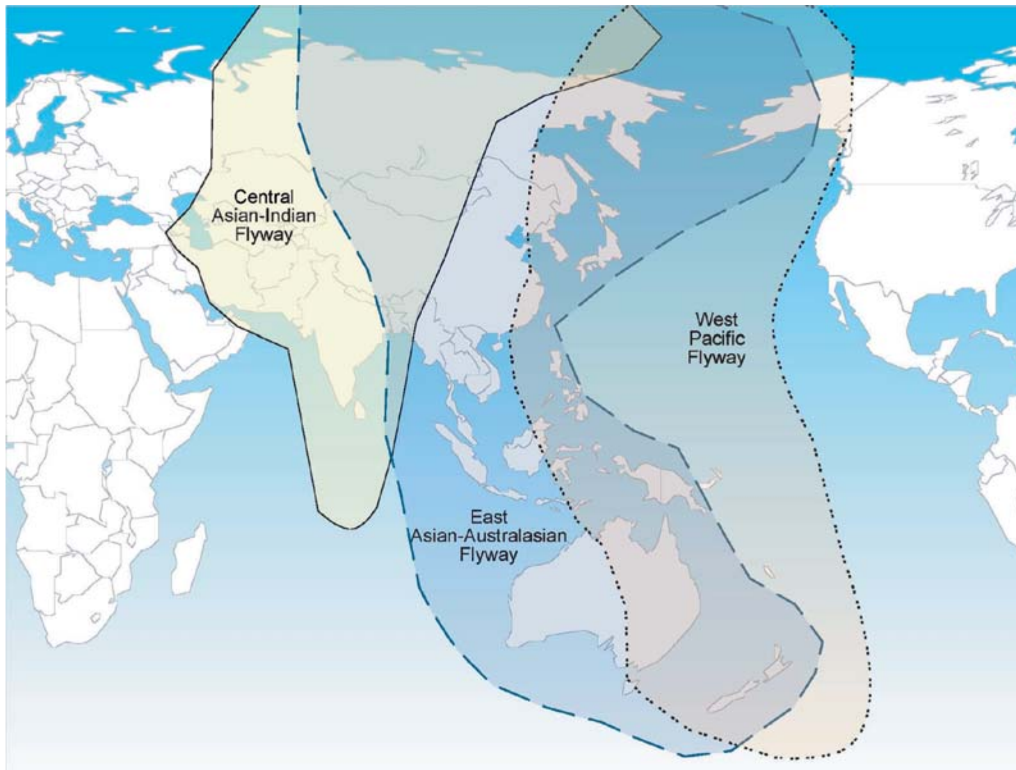

Fig. 2. Asian flyways for migrating birds

Little information currently exists about the influenza A virus reservoir in wild aquatic birds in Asia. One of the few surveillance studies that have been performed [29] reports the isolation of 9 HA subtypes and 8 NA subtypes during 1995 to 1998 in Siberia and Japan. Ducks were sampled from August to October, and shorebirds were sampled in August and September. Isolates were obtained from ducks only. Subtypes H2, H7, H10, H14, H15 and N5 were not detected, and no HPAI viruses were isolated from these wild ducks. The rate of isolation of influenza A viruses in this study was less than 2.0%.

Routine surveillance studies in ducks in New Zealand in 1997 [39] reported four isolates of H4N6 and two of H5N2. Also in 1997, epidemiologic studies indicated that wild aquatic birds might be the source for HPAI H7N4 in an outbreak in Australia [34]. Although no virus isolates were obtained, the most likely source of virus infection was from contaminated water from a river frequently used by wild waterfowl.

Some wild aquatic birds that can be classified as both resident and migratory for a specific region are also part of the influenza A virus reservoir. An example is the grey heron (*Ardea cinerea*), which maintains a resident population in Hong Kong SAR [4] and a migrant population in Southern China. The HPAI H5N1 virus recently has been isolated from these birds in Hong Kong [9].

The role of passerine birds in influenza transmission

The available evidence presented above is that aquatic birds are the main "players" in the maintenance of influenza A viruses in the world. However influenza A viruses of the majority of subtypes have been isolated at irregular intervals from caged birds from many countries and from a limited number of passerines, myna (*Acridotheres tristis*), and rarely from psitticine species (parrots) and pigeons [1]. Many of the isolates of influenza A virus from passerine birds were from imported dead birds at airports (customs) and the role of these influenza A viruses in disease is uncertain. The exception is the isolation of highly pathogenic H7N7 from starlings, an isolate associated with a highly pathogenic outbreak of avian influenza in Australia, with detection of antibodies in sparrows [26]. Experimental studies showed that both starlings and sparrows would support replication of the H7N7 virus and that transmission could occur from bird to bird. Overall the available information supports the notion that some passerine birds can support the replication of influenza A viruses but are probably not involved in perpetuating them. However, during outbreaks of influenza it is highly probably that these birds could be involved in local spread of influenza A viruses between poultry in houses that are not screened.

The H5N1 outbreaks in wild birds in Hong Kong in late 2002

In late November 2002 H5N1 virus was isolated from a dead little egret (*Egretta garetta*) and from a Canadian goose (*Branta canadensis*) in a nature park in Hong Kong [9]. In mid-December 2002 there was a second outbreak of H5N1 at another nature park in Hong Kong (Kowloon Park). The outbreak in Kowloon Park spread to all of the aquatic birds in the park and killed the majority of species [9, 41] including a wide range of exotic birds, such as greater flamingos (*Phoenicopterus ruber*) (Fig. 3). The virus causing this outbreak was first isolated from a grey heron. The noticeable features of these lethal H5N1 outbreaks in the parks were that each outbreak was caused by a different H5N1 genotype and that these genotypes had not previously been isolated from domestic avian species, implicating wild aquatic birds in the introduction of these viruses.

These two outbreaks were the forerunners of what was to come in Asia in 2003 and the viruses showed significant evolutionary changes compared to the 1997 H5N1 virus. The viruses were antigenically distinguishable from the earlier H5N1 strains and biologically much more pathogenic, killing a wide range of aquatic birds. Additionally one of the H5N1 genotypes transmitted to a single family in Hong Kong infecting two persons and killing one of them. The failure of this H5N1 virus to transmit to additional humans in Hong Kong was probably due to the strategies developed in Hong Kong to reduce the virus load in the poultry markets and minimize the possibility of transmission from poultry to humans (see below).

In addition to the isolation of H5N1 from wild birds mentioned above, the H5N1 virus was also isolated during 2003 from a black-headed gull (*Larus ridibundus*), from a feral pigeon (*Columba livia*), and from a tree sparrow (*Passer

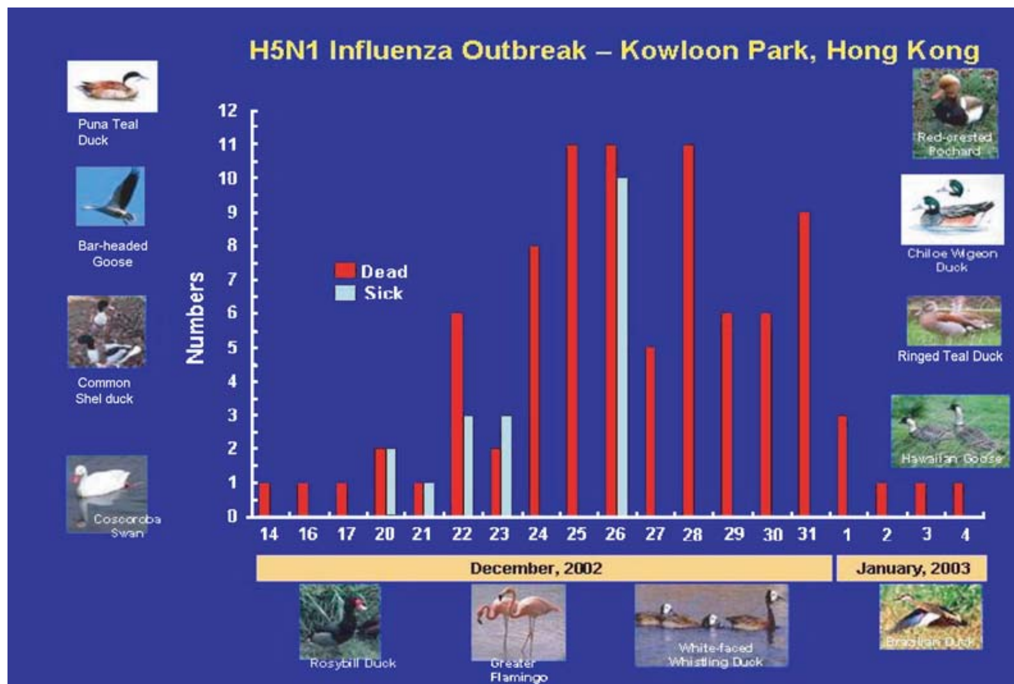

Fig. 3. H5N1 avian influenza A virus outbreak in Kowloon Park, Hong Kong SAR, China. Shown are the numbers of birds identified as sick or dead on each calendar day. H5N1 influenza A virus infection was confirmed by virus isolation from the affected birds. The species killed included Rosy-billed Pochard (*Netta peposaca*), Coscoroba Swan (*Coscoroba coscoroba*), Chestnut-breasted Teal (*Anas castanea*) Red-crested Pochard (*Netta rufina*), Chiloe Wigeon (*Anas sibilatrix*), Brazilian Teal (*Amazonetta basiliensis*), Greater Flamingo (*Phoenicopterus ruber*), Falcated Teal (*Anas falcata*), White-faced Whistling Duck (*Dendrocygna viduata*), Ringed Teal (*Callonetta leucophrys*), Common Shelduck (*Tadorna tadorna*), Hawaiian Goose (*Branta sandvicensis*), Bar-headed Goose (*Anser indicus*)

montanus). In early 2004 H5N1 was isolated from two dead peregrine falcons (*Falco peregrinus*) in Hong Kong. Prospective virological surveillance of other aquatic and passerine birds in Hong Kong during 2003 failed to detect H5N1 in other species. Thus H5N1 influenza A virus has been isolated intermittently from wild birds in Hong Kong and each isolate since 2003 has been the Z genotype.

Why was Hong Kong free of H5N1 in 2004?

It is notable that poultry in Hong Kong, SAR, China, remained free of H5N1 during the 2003–2004 epidemic. This is remarkable, especially since the above chronology indicates repeated outbreaks of H5N1 from 1997–2002. The reason is that Hong Kong authorities (Health Department, Agriculture and Fisheries, Environmental Services, University of Hong Kong) worked together to understand the ecology, epidemiology and molecular changes of the viruses and have modified animal husbandry and marketing practices to prevent H5N1 re-emergence. A summary of the changes include: (i) ***1998:*** Ducks and geese (the original source

of influenza genes) were banned from the live bird markets; they were killed and sold chilled; (ii) ***2000:*** "One clean day per month" was introduced, when all poultry markets are simultaneously emptied and cleaned; (iii) ***2001:*** Quail (a continuing source of influenza A virus genes) were removed from the markets; (iv) ***2002:*** Inactivated H5N2 vaccine was used to ring vaccinate poultry farms in Hong Kong where outbreaks of influenza had occurred and an additional clean day per month was introduced in the live bird markets; (v) ***2003:*** Vaccination was adopted on all poultry farms in Hong Kong along with improved biosecurity, and all farms in mainland China providing poultry to Hong Kong were required to vaccinate their poultry; (vi) Serological testing of samples of all batches of poultry entering the Hong Kong poultry market system were required to have H5N1 antibody titers of 40 or more in hemagglutination inhibition (HI) tests against A/Goose/Guangdong/1/96 (H5N1). Thus Hong Kong provides one strategy for the control of H5N1.

Concluding remarks

Did wild birds spread H5N1 across much of Eastern Asia? Let us first consider the other likely culprit – humans. The poultry industry is a huge integrated industry in Asia, with a number of firms having branches in China, Vietnam, Thailand, and Indonesia. Do people, poultry or fomites move viruses between these countries? In previous highly pathogenic outbreaks of H5 and H7 in multiple countries the spread was directly or indirectly attributable to humans. If humans were the spreaders in Asia we might expect that a single genotype might dominate. The above considerations indicate that indeed the Z genotype is dominant. However, while the Z genotype is dominant it is not the only genotype present in Asia. It is noteworthy that the dominant genotype in Indonesia is different from that in Vietnam and Thailand (unpublished), arguing against spread by humans to the entire region.

The actual dates of emergence of H5N1 in various countries have not been clarified; the "official" dates of January and February 2004 may not be when the first cases of H5N1 emerged in poultry. This is not the case with Japan; we know when and where these outbreaks occurred. The first outbreak of H5N1 in chickens occurred on January 11, 2004 in Yamaguchi, the second on February 16 in Onita, and the third on February 26 in Kyoto. These are widely separated places and with very strict quarantine and eradication procedures in place making it difficult to document human spread. Given that it is mid-winter at the time of this writing, it is difficult to credit wild migrating birds with the spread. However, H5N1 viruses were isolated from dead crows (*Corvus brachyrhynchos*) near infected chicken houses, suggesting that wild birds may be implicated in local spread.

The isolation of H5N1 virus from some of the wild birds in Hong Kong could be explained by scavenging from virus infected farms (e.g., black headed gull, tree sparrow, feral pigeon, egret) but it is more difficult to explain how grey herons were infected. Ornithologists tell us that grey herons are very shy of humans and feed on live fish. The dead herons in Hong Kong were isolated from fish farms –

a considerable distance from poultry farms. Regardless, one could argue that they became infected from contaminated water. It is more difficult to explain the infected peregrine falcon(s) – these birds catch live small animals on the wing for food, making it less likely that they were infected from domestic poultry or from contaminated food.

The spread of H5N1 over much of eastern Asia is consistent with a role for migrating birds, with local spread by humans or resident birds. From the above it is clear that much of the data is circumstantial and that serological and virological studies of wild birds in Asia and throughout the world are urgently needed to provide more definitive information.

The possibility that migrating birds are involved in the spread of the H5N1/04 virus in Asia has led to consideration of culling of migrating birds. This must be strongly discouraged, for it could lead to unknown ecological consequences. Influenza is a non-eradicable zoonotic disease that the poultry industry is learning to live with. Wild birds of all kinds must be kept out of poultry farms and chicken farms separated from duck and pig farms.

While the best option for the control of the H5N1/04 virus is depopulation of all infected birds this may not be feasible especially if the virus has become established in migrating birds. The different strategies being adopted in different countries, including culling or culling plus vaccination, suggests that it is likely this H5N1 virus will become endemic in poultry in Asia and will be a continuing threat to both animal and human health for the future. The prospect is that a vaccine for humans will eventually be needed, because H5N1 viruses will continue to evolve.

Note added in proof

Since this paper was written new information support the tenant that highly pathogenic H5N1 influenza virus is now endemic on waterfowl. The isolation of highly pathogenic (HP) H5N1 influenza virus from sick bar-headed geese (*Anser indicus*) with neurological symptoms at Qinghai Lake in western China confirms the circulation of HP H5N1 in long distance migrating birds. Over 1,500 bar-headed geese and smaller numbers of brown headed gull (*Larus brunnicephalus*), black headed gull (*Larus ichthaetus*) and great cormorant (*Phalacrocorax carbo*) died at Qinghai Lake in May 2005. Phylogenetic analysis of the genome of these H5N1 viruses from geese at Qinghai Lake reveals that they are of the Z genotype and are probably derived from domestic poultry in Southern China. In September 2005 surviving birds will migrate south to Myanmar and over the Himalayas to India. This could lead to a dramatic expansion of the range of HP H5N1 that will continue to threaten poultry and humans.

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Transient or occult HIV infections may occur more frequently than progressive infections: changing the paradigm about HIV persistence

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Summary. Evidence of transient HIV infections was found in 8 subjects at high-risk for HIV infection among 47 longitudinally studied over 2–5 (average ∼3.5) years, whereas only two subjects developed progressive infection. All of these subjects developed serum antibodies (Ab) to conformational epitopes of HIV gp41 (termed "early HIV Ab"), but the 8 transiently infected subjects lost this Ab within 4–18 months, and did not seroconvert to positivity in denatured antigen EIA or Western Blot (WB). However, the two progressively infected subjects eventually seroconverted in the EIA and WB tests within one to two months after the appearance of "early HIV Ab". HIV *env* and *nef* sequences were directly PCR amplified from the peripheral blood mononuclear cells (PBMCs) of two of the eight transiently infected subjects during the time of "early HIV Ab"-postivity, and these showed significant sequence divergence from the HIV strains in the laboratory, indicating that they were not laboratory contaminants. Genome identity typing ("paternity-typing") of PBMC samples obtained at the time of "early HIV Ab"-positivity, and later when Ab was absent from each of the 8 subjects, showed that blood samples were not mixed-up. This provides further evidence that transient or occult infection with HIV does occur, and perhaps at a greater frequency than do progressive infections.

The paradigm and the problem

Human viruses can be classified as either acute, existing in the host for only a short period of time, or persistent [1]. Usually, immune responses terminate acute viral infections by eliminating the virus and virus-infected cells from the host, while persistent viruses evade immune elimination. Few persistent viruses are always

persistent, however. For example, Hepatitis B virus persists in approximately 20% of infected people, and 80% have only acute infections [2]. Sixty–80% of people infected with Hepatitis C virus develop persistent infections, with 20–40% having only acute infections [3]. Adenoviruses and measles virus persist in only a small percentage of infected people [4, 5], while herpesviruses persist in most, if not all, infected people [6]. Most retroviruses establish persistent infections in their susceptible hosts, and HIV infections are both persistent and usually progressive, leading to the gradual loss of helper CD4+ T-lymphocytes and susceptibility to opportunistic infections. Although, induced immune responses appear to control Human Immunodeficiency Virus (HIV) burdens following acute infection, they apparently do not eliminate the virus. Nevertheless, a number of studies over many years have presented evidence that occasionally only the transient appearance of either HIV-specific immune responses or HIV itself in PBMCs, or both, occurs in some individuals at high-risk for HIV infection (infants born to HIV-positive mothers or in some adults at high-risk for HIV infection) [7–17]. There have been three different interpretations of these findings. One is that transient infections occurred, with the virus somehow being eliminated. Another is that HIV infection occurred and persisted, but did not progress and became occult (persistent but silent). A third interpretation is that these people became "exposed" to the virus (and thus produced an immune response) but were not infected (i.e., HIV did not replicate in the host) leading to use of the term "exposed, uninfected individuals". It is difficult to ascertain conclusively what is occurring in these cases. However, there is little precedent for the third interpretation. Usually, development of a specific immune response to any infectious agent means that an infection had occurred sometime in the past [18]. In fact, specific immune responses have been used for many years to diagnose which infections had occurred, because often the agent is difficult or impossible to detect directly.

This topic is very important, because it has not been entirely clear what is necessary for mounting an effective immune response to HIV and what will be necessary if an HIV vaccine is to be fully effective. If some infected individuals can naturally eliminate their HIV, it would be important to understand how this is occurring, because it could point the way to an effective vaccine or therapy.

Evidence for transient HIV infection

One of the earlier studies indicating the occurrence of transient HIV infection was by Imagawa and coworkers, who followed 133 high-risk individuals and detected virus by PBMC culture in approximately 20% of them. They were all still negative by Western blot [7]. Subsequent studies of these individuals found that the virus disappeared in most of them and could not be cultured any more [19]. The later report was confusing to most investigators at the time because it was unclear what was occurring. Looking retrospectively, it is possible that Imagawa and coworkers were detecting transient HIV infection in many of the high-risk people transiently HIV-culture positive. Many studies in the pediatrics AIDS field demonstrated that some babies born to HIV-positive mothers could possess HIV

or mount HIV immune responses for short periods of time [8, 20–22]. However, the reliability of these data was questioned in a study by Frenkel et al. [23]. That was a PCR and sequencing re-analysis of HIV sequences in stored PBMCs from 42 cases of infants previously reported to be transiently infected with HIV, and it indicated that extensive sample mix-ups or HIV DNA contamination had occurred in the earlier reported studies [23]. This casted doubt on most of the suspected cases of transient HIV infection.

Criteria for transient HIV infection

To convincingly demonstrate that transient HIV infections occur, rigorous criteria must now be met [23]. These criteria include: 1) demonstration that HIV can be detected in the subject's unmanipulated mononuclear blood or lymphoid cells; 2) demonstration by nucleotide (nt) sequence analysis that this HIV is not a laboratory contaminant; 3) demonstration that this virus is related at the nt sequence level to the HIV present in the source person, and 4) identification of PBMC samples by somatic markers [23]. We add another criterion: 5) demonstration that an HIV-specific immune response transiently occurs since, fundamentally, development of a specific immune response to an infectious agent usually means that infection by that agent had occurred at some time in the past. Since it can be very difficult to directly detect HIV early after infection, the inability to detect it directly does not necessarily mean that an infection had not occurred. It thus may be better to screen for transient infection by monitoring for an induced immune response, rather than for HIV sequences directly.

Transient immune responses

A great deal of work in the last decade has been focused on high-risk individuals who would be expected to be infected with HIV because of their high-risk behaviors, but do not become progressively infected. Studies of these individuals are considered important for understanding what may be effective for prophylaxis or treatment of HIV [24], and a number of studies have monitored for various factors that may be potentially important for HIV "resistance" in these subjects. These studies have led to the identification of a number of host genes which appear to either inhibit HIV infection or slow disease progression [24–27]. Many studies demonstrated that immune responses to HIV are often present in those subjects, which may be the basis for the apparent "resistance" to infection. Because of this, the term "exposed, uninfected" individuals has become widely used for these subjects. It is likely, however, that they had been transiently infected, which in turn is what induced the immune responses. If the latter is true, the initial induction of an immune response may be the reason for elimination of the virus. Those studies have shown that there are HIV-specific CTLs and CD4 cells reactive to HIV antigens in these subjects, as well as increased NK cell activity [22, 28–39]. A decrease in SDF1α-3′A polymorphism frequency, which would result in increased levels of SDF in plasma, and increases of several β-chemokines in serum have been reported in these type of subjects [27, 40, 41]. A general increase in

mucosal and systemic immune activities have been reported, as have increases in HIV neutralizing antibodies and decreases in Th-1 cytokine profiles [41–47]. Other studies have shown that PBMC from such subjects possessed increased resistance to HIV infection *in vitro* [48–50], and an increase in CD8 cells that produce an anti-HIV factor [51]. There are numerous studies showing the presence of HIV-specific IgA or IgG in various body fluids, and increased IFN-producing CD4 and CD8 cells in the blood of these subjects [52–59]. In some instances, HIV sequences had been transiently found [14–17, 60]. The question still remains, however, as to whether these immune responses were induced by transient infection or actually by exposure to HIV antigens without infection. Induction of immune responses only by antigen deposition on mucosal surfaces, and not due to infection itself, would be very unusual.

Evidence from other lentiviral infections

It may be informative to note that transient infections have been observed with other lentiviruses Feline Immunodeficiency Virus (FIV) and Simian Immunodeficiency Virus (SIV) [61–63]. With SIV, it appears that the route of infection and inoculum size are important in determining whether an infection will be progressive or transient. Intravenous inoculations of virus usually led to persistent infections, but transient infections appeared following mucosal inoculations. The smaller the inoculation dose, the greater the frequencies of transient infections [61]. Furthermore, evidence for FIV and SIV infections resulting in occult infections have also been reported [63, 64].

Our previous data

We previously reported evidence of transient HIV infection in two high-risk adults who were followed for 10–11 years after their transient infection episodes [16]. These two individuals transiently developed serum antibodies to conformational epitopes of gp41 and their PBMCs transiently contained HIV during the same time period. Antibodies reactive to conformational epitopes of HIV gp41 have been shown to be the first immune response usually induced following HIV infections, and these occur 2–8 weeks before antibodies that can react to denatured HIV antigens in EIAs or Western blots in infected subjects who progress in their infections [65, 66]. Genome identity typing (paternity typing) of PBMC samples of one of the subjects taken during the period of HIV-positivity and 10 years later when anti-HIV antibodies or HIV could not be detected showed that blood samples had not been confused. Thus, two tests that were independent of each other (serum Ab and HIV in PBMCs) demonstrated the transient presence of HIV infection in these individuals.

New data

To further study whether transient HIV infection occurs, we performed a prospective longitudinal analysis of a cohort of EIA-negative sexual partners of

Table 1. Longitudinal assessment for the presence of HIV-1 DNA and anti-HIV-1 antibodies in subjects at risk for HIV-1 infection

High-risk subjects	Blood code	Date	Genome identity	Nested PCR			Serum anti-HIV Ab			
				env	*gag*	*nef*	Live-cell IFA	Native gp160 EIA	Commercial EIA/WB	Neut
ST-03	HR-3	8-11-95		–			–		–	
	-25	11-10-95		–			–		–	
	-57	2-16-96		–			–	–	–	
	-95	6-25-96					–	–	–	
	-177	7-14-97		–			–	–	–	–
	-200	10-14-97		–			–	–	–	–
	-212	8-6-98		–	–	–	20	+	–	4
	-222	12-7-98	+	+	+	+	20	+	–	4
	-239	3-8-99		+/–	–	–	40	+	–	–
	-250	8-28-99	+	–	–	–	–	–	–	–
	-288	7-26-00		–	–	–	–	–	–	
ST-05	HR-5	8-15-95		–			–	–	–	–
	-22	11-2-95		+			40	+	–	12
	-53	2-1-96		+			640	+	+	
ST-08	HR-8	8-24-95	+	–	–	–	–	–	–	–
	-35	12-7-95		–	–	–	–	–	–	–
	-71	4-22-96		–	–	–	20	+	–	4
	-172	7-9-97	+	–			20	+	–	12
	-199	10-5-97		–	–	–	–	–	–	–
ST-10	HR-10	8-28-95		–			–		–	
	-30	11-30-95		–			–		–	
	-62	3-21-96		–			–	–	–	–
	-114	8-14-96					–	–	–	–
	-125	11-20-96		–			–	–	–	–
	-152	2-24-97		–		–	–	+	–	8
	-179	7-17-97		–		–	70	+	–	12
	-219	9-17-98	+	–	–	–	–	–	–	–
	-238	3-4-99		–	–	–	20	+	–	<4
	-252	8-28-99		–			–	–	–	–
	-261	12-9-99	+	–	–	–	–	–	–	–
	-275	3-24-00		–			–		–	–
	-290	8-11-00		–			–		–	–
ST-16	HR-18	9-28-95		–	–		–		–	–
	-43	1-4-96		–	–		–	–	–	–
	-68	4-9-96		–	–		–	–	–	–
	-98	7-15-96		–	–	–	20	+	–	10
	-120	10-9-96	+	+/–	+/–	+/–	80	+	–	10
	-160	4-21-97		–	–	–	80	+	–	12
	-176	7-14-97		+/–	+/–	–	80	+	–	10
	-197	10-6-97		–	–		40	+/–	–	4
	-201	1-5-98					20	+/–	–	–

(*continued*)

Table 1 (*continued*)

High-risk subjects	Blood code	Date	Genome identity	Nested PCR			Serum anti-HIV Ab			
				env	*gag*	*nef*	Live-cell IFA	Native gp160 EIA	Commercial EIA/WB	Neut
	-211	7-20-98		–	–	–	–	–	–	–
	-224	10-19-98					–	–	–	–
	-232	1-19-99					–		–	–
	-243	4-20-99	+	–	–	–	–	+/–	–	–
ST-51	HR-124	11-12-96	+	–	–	–	20	+	–	12
	-210	7-23-98		–	–	–	40	+	–	4
	-218	12-11-98		–	–	–	20	+/–	–	–
	-246	8-23-99	+	–	–	–	–	–	–	–
	-287	7-12-00		+			1280	++	+	4
	-289	8-8-00							+	
ST-55	HR-137	1-7-97		–			10	+/–	–	+
	-159	4-10-97		–			–	–	–	–
	-173	7-10-97	+	–	–	–	40	+	–	+
	-199	10-14-97		–			–	–	–	–
	-215	9-28-98		–			20	+	–	–
	-228	12-16-98		–	–	–	20	+	–	–
	-254	10-19-99	+	–	–	–	–		–	–
	-273	3-21-00		–			–	–	–	
	-285	6-16-00		NT			–		–	
B-52	52.1	7-28-98	+	+	+	+Δ	40	+	–	10
	52.2	12-9-98		–	–	–	–	–	–	–
	52.3	5-18-99		–	–	–	–	–	–	–
	52.4	3-21-00	+	–	+	+	40	+/–	–	4
	52.5	5-24-00	+	–	–	–	–	–	–	–
B-13	13.1	4-14-98	+	–	–	–	20	+	–	–
	13.2	12-9-98		–	–		–	–	–	–
	13.3	5-19-99	+	–	–		–	–	–	–

PBMCs throughout were isolated from high-risk subjects' blood samples by density centrifugation in Ficoll-Hypaque, and the isolated PBMCs and plasmas or serum were stored at −70 °C until used. The sera or plasma were tested for antibodies to native *env* antigens by live cell IFA analyzed on a flow cytometer and by native gp160 EIA (66), by the Abbott HIV EIA, and by an HIV neutralization assay. The reciprocal of the serum or plasma dilution which immuno stained 50% of the cells or reduced the titer by 50% (neutralization) is presented as the titer

PBMCs samples were sent to Lab Corporation, Inc. for genome identity typing using 9 different polymorphic markers. + and + for a pair of samples indicates that each sample came from the same person (probability of mis-identity $\sim 2 \times 10^{-11}$)

Nested PCR for 3 different regions of the HIV genome were performed on DNA extracted from PBMCs

HIV-1-infected patients to look for evidence of transient or occult infections. At three to six month intervals, blood was drawn from each subject, processed and cryo-preserved following standard procedures, and serum or plasma screened for early anti-gp41 conformation-specific antibodies by live cell-IFA [65] and

a native gp160 third-generation EIA [66]. The PBMCs were screened for HIV proviral DNA by nested PCR-amplification of env, *nef* and *gag* sequences. A total of 47 individuals were followed for 2–5 years, and eight of them showed indications of transient infection, transiently possessing "early gp41 antibodies", which disappeared 4–18 months later (Table 1). Two of the 47 subjects developed progressive infections, eventually became positive in the denatured antigen EIA and Western blot tests (Table 1). This gave a frequency of about one progressive infection/100 subjects/year. This fits with published estimates of the frequencies of EIA/WB seroconversions within discordant-partner high-risk groups [67]. However, transient "early HIV Ab"-positivity occurred at a frequency of ~17% (8/47) over 2–5 years or ~3–4%/yr. If the latter are true infections that were naturally contained, then HIV may be more like Hepatitis B Virus, which persists in fewer than half of infected humans.

Characterizations of HIVs

We were able to directly amplify HIV sequences in the unmanipulated PBMCs by nested PCR in two of the cases who had transient early HIV Ab (ST-03 and B-52). During the first three years of visits to the clinic, subject ST-03 did not present with any serum Ab to HIV nor any evidence of proviral DNA in his PBMCs by nested PCR. He then tested positive for three consecutive blood draws over 7 months for "early HIV antibodies" to gp41, and HIV *env*, *gag*, and *nef* sequences in his PBMCs were amplified in one of these blood draws by nested PCR (Table 1). However, HIV DNA and serum antibodies could not be detected at later time points over the following 18 months. The amplified HIV *env* (v1-v3), *nef* and *gag* (coding for p17 matrix) sequences were analyzed by Blast search (NCBI, NIH) and showed maximum sequence identities of 92%, 94%, 94% at the nucleotide (nt) level respectively to the reported HIV sequences in the database. This virus is quite unique and the divergence of its nt sequences from common laboratory strains (Fig. 1) demonstrates that this virus was in the PBMC's collected from this individual, rather than a contaminant resulting from (or "originating as") commonly used laboratory HIV strains during sample processing and/or PCR.

To rule out the possibility of mislabeling of blood samples collected at different time points, genome identity testing (paternity typing) was performed by Lab Corp., USA, on a pair of PBMC samples from ST-03 (Table 1). Nine independent polymorphic loci in each sample were analyzed. The analysis confirmed that the PBMCs obtained during the time of Ab- and HIV-positivity and those taken 2 years later when the subject was Ab- and HIV-negative were from the same person (probability of mis-identity was calculated to be on the order of $\sim 2.9 \times 10^{-11}$). This case thus meets four of the five criteria discussed above in order to convincingly document transient HIV infection.

Subject B-52 showed the presence of "early anti-gp41 antibodies" and proviral DNA in his PBMCs at his initial visit, but these disappeared within the following four months. Both anti-gp41 antibodies and HIV DNA (*gag* and *nef*) in his PBMCs appeared again 2 years later (fourth visit), but the *env* region could not be amplified

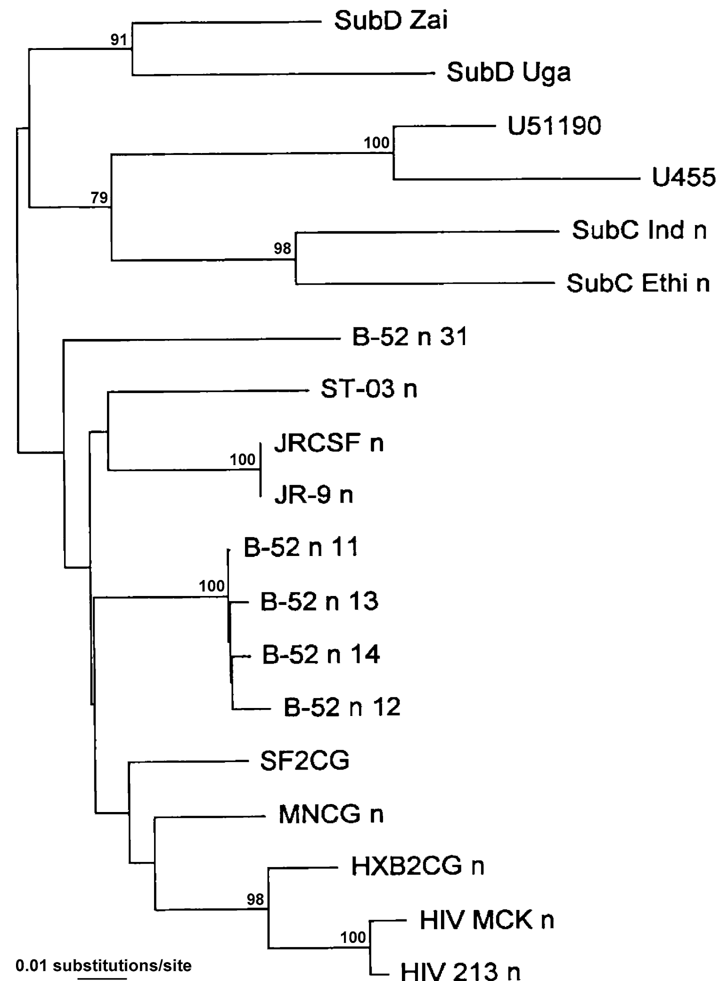

Fig. 1. Phylogenetic analyses for *nef* sequences derived from ST-03 and B-52: Standard sequences were included as follows; *HIV Subtype A*: U51190 and U455; *HIV Subtype B*: JRCSF n, JR-9_n, SF2CG, MNCG n, HXB2CG n, HIV MCK n, HIV 213 n. (all of these exist as HIV strains in the lab). *Nef* sequence derived from ST-03: ST-03 n; *nef* sequences derived from B-52 (1st visit): B-52_n_11, B-52_n_12, B-52_n_13, B-52_n_14, and *nef* from B-52 (3rd visit): B-52_n_31

from his PBMCs (Table 1). On his initial visit, only 35 cycles of first round PCR could amplify HIV DNA, but the amplified *nef*-band was found to be shorter in length than expected. However, the deleted *nef* could not be seen on his fourth visit and a *nef* region of only expected size was amplified. In the following two visits, none of the three regions (*env*, *nef* and *gag*) could be amplified, indicating that he was able to clear HIV from his circulation. HIV *env* and *gag* sequences amplified on the first visit showed the closest degree of sequence identify (∼95%) with HXB2 (the original molecular clone of HIV). However, the deleted *nef* region showed 92% identity at the nt-level with the same strain, HXB2. To rule out the possibility that the amplified *nef* region may represent a different HIV strain

in the same sample, we amplified by PCR almost 4kb of HIV DNA spanning nearly the entire 3′-half of the HIV genome, which includes *env*, *vpu* and *nef* regions. The analyses of *env*, *vpu* and *nef* regions of the same clone showed 95%, 94%, and 92% sequence identity to the respective regions of HXB2, which is in agreement to the nt homology found when *env* and *nef* regions were amplified separately. This argues against the possibility that the PCR-amplified HIV regions in this case was an artifact of contamination from a laboratory strain, such as HXB2, as the analyzed regions showed different pattern of nt sequence homology to this HIV strain. On his fourth visit, sequence analysis of the PCR-amplified *nef* band of expected size showed about 9% and 11% dissimilarity at the nucleotide level with the *nef* sequence detected on his first visit and that of strain HXB2, respectively. The differences in nucleotide sequences (Fig. 1) reveals that he was probably infected with different HIV strains at different times, as he was known to have been exposed to multiple HIV-seropositive sexual partners. However, he was apparently able to "handle" the virus each time.

Discussion

These data, demonstrating that some people at high-risk for HIV infection transiently develop HIV-specific antibodies for 4–18 months, adds to a large number of reports showing transient immune responses in high-risk. These immune responses are not seen in subjects at low risk for HIV infections. Further, since HIV also can be detected directly in a subset of these subjects, it is very likely that they had been infected with HIV. However, why the virus did not persist in those individuals, in contrast to those who develop progressive infections, is not clear.

Until now, other than known genetic polymorphisms that restrict HIV infection or spread within the body, immune responses such as HIV-specific cytotoxic T-lymphocyte response and non-cytotoxic T cell-mediated viral inhibition have been postulated to explain resistance of persistently "resistant" high-risk people. Specifically, the presence of HIV-specific cytotoxic T-cells have been found in the blood of children born to HIV-positive mothers and in "exposed", high-risk, adults seronegative by standard denatured antigen antibody tests [20, 21–28, 30–38]. Furthermore, the presence of major histocompatibility complex (MHC) class I-restricted CTLs have been reported by some groups to indicate that HIV infection occurred with at least one or more rounds of replication *in vivo*. An important tenet in infectious disease studies is that induction of an immune response specific to an infectious agent usually means that an infection with that agent had occurred [18]. Thus, based on serologic criteria, we identified 8 new subjects transiently infected with HIV among 47 individuals longitudinally studied, giving a frequency of about 17% (8/47) over a 3.5 year average period, or ∼4–5% per year. This frequency may have been higher if blood samples had been collected at more frequent time points, since some transient infections may have been missed during the intervals between samplings. Still, it is unclear whether a CTL response or a non-cytotoxic CD8+ response is solely responsible for long-term protection of

high-risk individuals from persistent HIV infections, despite their high level exposure to HIV [33, 68]. The presence of HIV-neutralizing Abs in sera of transiently infected people (Table 1) could help inhibit the spread of the virus at early stages of infection. Studies in macaques showed that prior exposure to "sub-infectious" doses of live SIV, either intravenously or intrarectally, provided protection to these animals when higher infectious doses were administered intrarectally a few months later [69]. These animals did not seroconvert in tests with denatured antigens but possessed CMI, and they likely had neutralizing antibodies. However, they showed transient PCR positivity for SIV DNA in their PBMCs, and therefore were probably transiently infected. It would be interesting to see if these animals would be positive in the native gp160 EIA for SIV "early antibodies" during their transient PCR-positivity phase. Together, these responses could limit HIV spread by neutralizing viral particles and killing new virus-infected cells, thereby providing protection from (establishing) HIV-related disease. Recently, Rosenberg et al. [70] have shown that containment of viral replication can be achieved, when virus-specific CTL and T-helper activity were maintained at certain minimal levels even after patients were not treated with drug therapy for at least six months. A vaccine that preserves native conformational epitopes of gp160 and elicits neutralizing antibody and a CTL response together might be promising for the most effective immunoprophylaxis.

Conclusions

The data presented here strongly suggest that transient or occult HIV infection occurs in some high-risk adults. Understanding the reason(s) behind this type of outcome will undoubtedly be beneficial for development of either an effective vaccine or new therapy.

Materials and methods

Isolation of PBMCs and serum

PBMCs were isolated from high-risk subjects' blood samples by the Ficoll-Hypaque method. Isolated PBMCs and plasma or serum were stored in liquid nitrogen and at −70 °C, respectively, until use.

Live-cell indirect immunofluorescence assay (live-cell IFA)

Live HIV-infected and uninfected H9 cells were immunostained with serially diluted plasma or sera collected from high-risk subjects, and subjected to flow cytometric analysis. The reciprocal of the serum or plasma dilution that stained 50% of the cells was considered the titer.

Isolation of PBMC-DNA and polymerase chain reaction (PCR)

PBMCs were resuspended in a buffer (10 mM Tris [pH 7.8], 10 mM EDTA and 0.5% sodium dodecyl sulphate [SDS]) and treated with 200 μg of Proteinase K per ml overnight at 42 °C. DNA was isolated using phenol-chloroform extraction followed by ethanol precipitation and finally dissolved in PCR-grade water. Nested PCR for V1–V3 of *env*, *gag*, and *nef* regions were performed as reported by Zazzi et al. [71].

Native gp160 EIA

Purified native gp160 derived from HIV-1_{IIIB}-infected H9 cells was obtained from Advanced Biotechnologies. Native gp160 was coated onto flat-bottom PRO-BIND EIA plates (Falcon) and the EIA performed as described in Ref. 66. More than 50 serum or plasma samples from low-risk university personnel were used side by side as controls at the same dilution (1:4). Duplications of each sample were used, and every test was repeated at least twice. The cutoff value was defined as twice the optical density (OD) value obtained with 50 random low-risk personnel samples. Results were expressed as signal-to-cutoff ratio (s:c), and an s:c ratio >1 was considered reactive.

HIV neutralization assay

To determine the neutralizing antibody titer of the serum or plasma, 25 μl of three-fold serial dilutions were mixed with 25 μl of HIV_{213} containing 75 Fluorescent Forming Unit (FFU) of infectious HIV, and incubated for one hour at 37 °C to allow antibody to neutralize infectivity of HIV. Twenty-five μl of the mixture was then added to each of two replicates of Ghost-CXCR4 cells obtained from the NIH-AIDS Repository. The cultures were incubated for 2 days and the number of fluorescent cells (typically 75 FFC) recorded. The 50% HIV neutralizing endpoint ($p < 0.05$ by the student's t-Test) is the reciprocal of the highest dilution of sample that resulted in a 50% reduction of the number of fluorescent Ghost-CXCR4 cells in comparison with a negative serum control.

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Ehrlichia under our noses and no one notices

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Summary. *Ehrlichia chaffeensis*, an obligately intracellular bacterium, resides within a cytoplasmic vacuole in macrophages, establishes persistent infection in natural hosts such as white-tailed deer and canids, and is transmitted transstadially and during feeding by ticks, particularly *Amblyomma americanum*. Ehrlichial cell walls contain glycoproteins and a family of divergent 28 kDa proteins, but no peptidoglycan or lipopolysaccharide. The dense-cored ultrastructural form preferentially expresses certain glycoproteins, including a multiple repeat unit-containing adhesin. Ehrlichiae attach to L-selectin and E-selectin, inhibit phagolysosomal fusion, apoptosis, and JAK/STAT activation, and downregulate IL-12, IL-15, IL-18, TLR2 and 3, and CD14. Mouse models implicate overproduction of TNF-α by antigen-specific CD8 T lymphocytes in pathogenesis and strong type 1 CD4 and CD8 T lymphocyte responses, synergistic activities of IFN-γ and TNF-α, and IgG2a antibodies in immunity.

Human monocytotropic ehrlichiosis (HME) manifests as a flu-like illness that progresses in severity to resemble toxic shock-like syndrome, with meningoencephalitis or adult respiratory distress syndrome in some patients, and requires hospitalization in half. In immunocompromised patients, HME acts as an overwhelming opportunistic infection. In one family physician's practice, active surveillance for three years revealed an incidence of 1000 cases per million population. Diagnosis employs serology or polymerase chain reaction, which are not utilized sufficiently to establish the true impact of this emerging virus-like illness.

Introduction

Acute febrile syndromes with headache, myalgia, and other systemic symptoms which the patient must suffer and endure without effective therapy directed at the causative agent are caused by a large group of viral and other diseases. The prevalence of influenza viruses lends its name to the condition, namely flu-like illness. In many tropical regions a common clinical diagnosis is dengue fever. When closer scrutiny is applied, many other viral and bacterial agents are identified in patients diagnosed as having dengue fever. It is particularly discouraging to

observe that rickettsial and ehrlichial diseases, which can be treated effectively with tetracycline antimicrobial drugs, are so consistently neglected. This is especially true for *Ehrlichia chaffeensis* infection, which leads to hospitalization of half of the infected patients and death in 3% of them [10, 16, 31, 35].

The organism

Agents related to *E. chaffeensis* had been identified and studied by veterinary scientists since 1908, when Theiler in South Africa identified *Anaplasma marginale*, a tick-transmitted obligately intracellular bacterium that parasitizes bovine erythrocytes. Subsequent veterinary discoveries included *Ehrlichia* (formerly *Cowdria*) *ruminantium* by Cowdry in 1925, *E. canis* by Donatien and Lestoquard in 1935, *A. phagocytophilum* by Gordon in 1940, and *E. ewingii* by Ewing in 1971.

A case of human ehrlichiosis associated epidemiologically with tick bites in Arkansas was reported in 1987 based upon observations of the ultrastructural appearance of small bacteria in vacuoles of circulating monocytes and the development of antibodies reactive with *E. canis*; *E. canis* was subsequently recognized to share antigens with the etiologic agent, *E. chaffeensis* [26].

Ehrlichia and *Anaplasma* are related genera of the family Anaplasmataceae, which also includes *Neorickettsia* and *Wolbachia* [13]. Among the related bacteria that appear to infect humans, *E. chaffeensis* is more pathogenic than is *A. phagocytophilum*, *E. ewingii*, *E. muris*, and *E. canis* [6, 31, 38].

Ehrlichiae manifest two ultrastructural appearances, a larger reticulate cell and a smaller dense-cored form [39]. Some proteins, such as a glycoprotein that contains 2 to 5 identical hydrophilic tandem repeat units, are preferentially expressed on the dense-cored form and/or are secreted into the vacuole within which the bacteria divide by binary fission [28, 40]. Ehrlichiae possess a substantial variety of surface-exposed glycoproteins and a prominent multigene family of surface-exposed proteins of 26–30 kDa [8, 33, 52]. These p28s are complete genes in a locus on the chromosome. The p28 locus of *E. chaffeensis* encodes 22 related proteins that differ greatly in three hypervariable regions that are predicted to be surface-exposed. DNA sequence analysis of particular genes of numerous strains reveals genetic divergence along three lineages, with intralineage divergence also and wide geographic dispersal of all three lineages [9, 51]. These observations support the concept of a long evolutionary history of *E. chaffeensis* in North America. In contrast, *E. canis* is virtually clonal in North America, suggesting introduction in relatively recent times and minimal subsequent evolutionary divergence.

The cell wall structure of *Ehrlichia* is distinct from those of *Rickettsia* and *Orientia* at both the ultrastructural and molecular levels [39]. *Ehrlichia* possess neither lipopolysaccharide nor peptidoglycan, resulting in a fragile organism that is difficult to purify in a stable infectious state. It has been hypothesized that disulfide bonds linking cell wall proteins are important in ehrlichial structural integrity [29].

Epidemiology

Ehrlichia chaffeensis has been identified in nature in white-tailed deer (*Odocoileus virginianus*), coyotes (*Canis latrans*), dogs, goats, lone star ticks (*Amblyomma americanum*), and American dog ticks (*Dermacentor variabilis*) [5, 20, 21, 45]. Experimental and epidemiologic evidence indicate that persistently infected white-tailed deer are the principal reservoir and that lone star ticks are the main vector [14]. The increasing prevalence of *E. chaffeensis* has been associated with the appearance of an increasing population of *A. americanum* ticks, for which deer are a frequent host. The ticks do not transmit *E. chaffeensis* transovarially from one generation of ticks to the next, but do acquire ehrlichiae during the blood meal taken as a larva or nymph. Ehrlichiae are maintained transstadially as the infected larva or nymph moults [14].

The highest incidence of *E. chaffeensis* infection of human monocytotropic ehrlichiosis (HME), occurs in the geographic distribution of *A. americanum* ticks from New Jersey to Kansas and southward through the southeastern and south central states [10, 16, 31]. Cases of HME also occur in states outside of this distribution, presumably owing to transmission by *D. variabilis* and to travel-acquired cases. HME has been reported in 47 states.

Passive reporting suggests that HME is a rare disease (5 cases per million population in Arkansas during a period when this state reported the highest incidence of all states). However, active surveillance has determined much higher incidence [7, 35]. In one primary care physician's practice, an incidence of approximately 1000 cases per million population was documented by strict laboratory confirmation criteria for three consecutive years [35]. It is very likely that the latter rate is closer to reality in the vast rural and suburban areas of the south central and southeastern US where white-tailed deer and lone star ticks abound.

HME cases occur between April and September with 68% occurring in May–July [16]. The median age (44–51 years) is substantially older than that of Rocky Mountain spotted fever patients, suggesting even greater importance of age-dependent host factors in the severity of illness. There is a strong male predominance among diagnosed cases, similarly suggesting gender-dependent host factors in disease severity [16, 31, 35].

Clinical manifestations

The signs and symptoms of HME described in a well documented series of cases diagnosed at the Centers for Disease Control and Prevention included fever (97%), headache (81%), myalgia (68%), anorexia (66%), nausea (48%), vomiting (37%), rash (36%), cough (26%), pharyngitis (26%), diarrhea (25%), lymphadenopathy (25%), abdominal pain (22%), and confusion (20%) [16]. More than 60% were hospitalized. An active, prospective population-based study in Cape Girardeau, Missouri observed similar signs and symptoms, with more than 40% requiring hospitalization, indicating that HME is a relatively severe, multisystem disease [35]. Severity has been compared with Rocky Mountain spotted fever and toxic

shock syndrome. Meningoencephalitis and adult respiratory distress syndrome have been documented in significant portions of cases [17, 44].

In addition to posing a life threatening risk to apparently immunocompetent persons, *E. chaffeensis* can act as an overwhelming opportunistic infection in immunocompromised persons, such as those with acquired immunodeficiency syndrome, those under corticosteroid treatment, survivors of cancer chemotherapy, and allograft transplant recipients [37, 41]. In comparison with only few scattered organisms in the tissues of fatal illness in immunocompetent persons, suggesting immunopathogenesis, the tissues of immunocompromised patients contain massive quantities of ehrlichiae, a clear failure of the immune system to control the infection.

Prominent clinical laboratory data are leucopenia, thrombocytopenia, and elevated concentrations of hepatic transaminases in most patients [16, 35].

A prospective study of tick-exposed soldiers at Ft. Chaffee, Arkansas demonstrated the occurrence of seroconversion in some subjects who reported no symptoms during the period of the investigation [49]. It seems more likely that these individuals developed antibodies that were crossreactive with *E. chaffeensis* after being bitten by a tick carrying a less pathogenic organism, such as *E. ewingii* or the unnamed *Anaplasma* species that is highly prevalent in white-tailed deer [32, 45]. Indeed, human infections with *E. ewingii* have been reported predominantly in immunocompromised patients, none of whom have died [6]. The likelihood of asymptomatic infection with *E. chaffeensis* would seem remote.

Pathogenesis

Ehrlichia chaffeensis is introduced into the skin when the tick takes a blood meal and spreads systemically, infecting monocytes and macrophages in the blood and tissues. The pathologic lesions include perivascular infiltration of lymphocytes and macrophages, some of which are infected with *E. chaffeensis*, hepatocellular apoptosis, granulomas, and diffuse alveolar damage in the lungs [47]. Perivascular inflammation in the central nervous system is the basis for the meningoencephalitis. Bone marrow biopsies, presumably obtained to evaluate leucopenia, thrombocytopenia, and/or anemia, reveal hyperplasia and megakarocytosis (appropriate compensatory responses), erythrophagocytosis, and granulomas [12]. The presence of erythrophagocytosis suggests that peripheral sequestration and destruction of leukocytes, platelets, and red blood cells may be the mechanism of the cytopenias. The granulomas likely play a role in the immune clearance of ehrlichiae, as they are also observed in the murine *E. muris* animal model at the time of immune control of the infection [36].

The tandem repeat-containing glycoprotein of *E. chaffeensis* is an adhesin, although other ehrlichial adhesins might also exist [40]. The host cell receptors for *E. chaffeensis* are L-selectin and E-selectin [53]. Once within the host cell, *E. chaffeensis* resides in a membrane-bound vacuole where it grows and manipulates the host cell to the ehrlichia's advantage. Ehrlichiae inhibit phagolysosomal fusion in association with decreased expression of vesicle-docking molecules (SNAP23,

Rab5A, and syntaxin 16) and prolong the life of the host cell by induction of apoptosis inhibitors (NF-κB, IER3, BirC3, Bcl2 and MCL 1) and downregulation of expression of apoptosis inducers (BiK and BNIP3L) [54]. Ehrlichiae redirect transferrin receptors containing iron to the ehrlichial vacuole for their utilization [3, 4].

Ehrlichia chaffeensis is a stealth organism that has evolved over millions of years to survive in phagocytic host defense cells. They downregulate expression of key host defense molecules including IL-15, IL-18, chemokine receptors 2, 3, and 4, and major histocompatibility class II [54]. The ehrlichiae avoid activating IL-12, Toll-like receptors 2 and 3, and CD14, and they inhibit the JAK/STAT pathway of macrophage activation [2, 22, 25].

Two mouse models of monocytotropic ehrlichiosis have revealed different host-pathogen interactions resulting in lifelong persistent infection in *E. muris*-infected mice with absence of disease and in toxic shock-like illness resembling fatal HME in mice infected with an unnamed *Ehrlichia* obtained from *Ixodes ovatus* ticks (IOE) [19, 36, 42, 43]. The two *Ehrlichia* species are closely related genetically and antigenically. IOE causes disseminated infection of monocytes and macrophages, severe hepatic necrosis and apoptosis, interstitial pneumonia, leucopenia, thrombocytopenia, and death [43]. The fatal course is associated with an early elevation and overproduction of tumor necrosis factor-α by antigen-specific CD8 T lymphocytes and a late high concentration of serum IL-10 [18]. CD8 gene knockout mice survive IOE infection. Neutralization of TNF-α does not result in survival of IOE-infected mice, which develop an earlier rise in IL-10 and much higher quantities of IOE. These observations support the pathogenetic concepts of cytokine-mediated sepsis and immunosuppression.

Immunity

The relatively non-pathogenic *E. muris*-mouse model delineated the importance of CD8 and CD4 T lymphocytes, cytotoxic T lymphocyte activity, synergistic activities of gamma interferon and tumor necrosis factor-α, and antibodies in the control of ehrlichial infection [15]. Earlier studies by Winslow and colleagues had identified epitopes on the first hypervariable region of p28–19 as targets of antibody-mediated protective immunity against *E. chaffeensis* in SCID mice [23, 24, 48].

Ismail et al. investigated the highly pathogenic IOE-mouse model, demonstrating by adoptive transfer that immune CD4 and CD8 T lymphocytes and polyclonal antibodies from *E. muris*-immune mice provided cross-protection against IOE [18]. In the model in which *E. muris*-infected mice were infected with an ordinarily lethal dose of IOE, cross-protection was associated with a strong type I CD4 and CD8 T lymphocyte response and a substantial memory type IgG2a response. Elucidation of the protective immune effectors and development of vaccines against ehrlichiae pose a stiff challenge for which the tools are now becoming available.

Diagnosis

Establishment of the epidemiologic and clinical importance of *E. chaffeensis* and timely administration of effective antimicrobial treatment ultimately depend upon better education of physicians and the public, appropriately enhanced clinical suspicion of the diagnosis, and availability and utilization of laboratory diagnostic methods. Currently the extent of use of empiric treatment with doxycycline is not known, and sending of blood and/or acute and convalescent serum samples to referral laboratories is not commonly practiced [34].

The gold standard diagnostic test is immunofluorescent antibody assay using *E. chaffeensis*-infected cell culture as the test antigen [46]. The method is available commercially, but interlaboratory standardization of the method has yet to be achieved. The majority of patients have not developed detectable antibodies at the time of presentation for medical attention, requiring testing of convalescent sera for the development of antibodies or a rising antibody titer [11]. Commercial laboratories hardly ever receive more than a single serum sample from any patient [34]. Thus, the criteria of seroconversion, a four-fold rise in titer, or a single titer of 256 or greater are not achieved in many patients whose true diagnosis is HME. A portion of healthy persons' sera contains antibodies reactive with *E. chaffeensis*, although not necessarily stimulated by this ehrlichia. Several recombinant proteins offer the opportunity for a standardized, commercially available method [30, 50].

In the acute stage of HME, leukocytes containing cytoplasmic vacuoles filled with ehrlichiae, so-called *morulae* (Latin = mulberry) because of their resemblance of the microcolonies to this fruit, are visible in the peripheral blood in approximately 10% of patients. Morulae are more abundant in immunocompromised patients, detectable in half of these cases. Search for morulae is tedious, laborious, and prone to confusion with Döhle bodies, overlying platelets and contaminating particles, thus requiring specialized experience.

Isolation of *E. chaffeensis* is a research tool that is generally unavailable for clinical use and requires several days for ehrlichial detection under the best of circumstances. Polymerase chain reaction is sufficiently sensitive to serve as a useful clinical diagnostic method. Several gene targets have been demonstrated to be effective. The two major problems have been test availability and DNA amplicon contamination. Real time PCR with all appropriate precautions and controls offers the possibility of a timely diagnostic result from a reference laboratory. The prospects for what is really needed, namely a simple, inexpensive, sensitive, specific point-of-care test, are presently only a dream.

The time when HME will be recognized as important as, or more important than, Lyme borreliosis and West Nile virus infection in the US is likely still a decade in the future.

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The role of reverse genetics systems in determining filovirus pathogenicity

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Summary. The family *Filoviridae* is comprised of two genera: *Marburgvirus* and *Ebolavirus*. To date minigenome systems have been developed for two Ebola viruses (*Reston ebolavirus* and *Zaire ebolavirus* [ZEBOV]) as well as for *Lake Victoria marburgvirus*, the sole member of the *Marburgvirus* genus. The use of these minigenome systems has helped characterize functions for many viral proteins in both genera and have provided valuable insight towards the development of an infectious clone system in the case of ZEBOV. The recent development of two such infectious clone systems for ZEBOV now allow effective strategies for experimental mutagenesis to study the biology and pathogenesis of one of the most lethal human pathogens.

Introduction

Reverse genetic systems can be broadly grouped into two categories: minigenome and infectious clone systems. Both utilize cloned cDNA to either mediate expression of reporter genes (minigenome systems) or to produce infectious virus (infectious clone systems). Together they provide excellent tools for studying replication and transcription as well as infectivity and pathogenicity. The first reverse genetic systems were established for positive-sense, single-stranded RNA viruses [62, 73], where transfection of the full-length genomic RNA transcripts into eukaryotic cells resulted in viral protein expression, viral replication, particle formation, and release. The development of reverse genetics systems for these viruses was favored by the fact that the genomic RNA of positive-sense RNA viruses can directly serve as the template for the expression of viral proteins through the cellular machinery. In contrast, negative-sense RNA viruses first need to transcribe their genomes into positive-sense RNA prior to translation of viral proteins, a step that is dependent on the presence of a functional viral replicase complex, since cells lack the necessary enzymes to mediate $(-)\text{RNA} \rightarrow (+)\text{RNA}$ synthesis.

In the past decade, several reverse genetic systems have been developed for negative-sense RNA viruses [55], with the establishment of a minigenome system generally preceding the development of the infectious clone system, although this is not always the case. Reverse genetics systems have been developed for representatives of the negative-strand RNA virus families *Orthomyxoviridae*, *Bunyaviridae*, *Arenaviridae*, *Rhabdoviridae*, *Paramyxoviridae*, *Filoviridae* and *Bornaviridae*, either in the form of minigenome systems and/or infectious clone systems. In each case the technology used reflects both the particular requirements of the virus as well as the availability of established methodologies.

The first negative-sense RNA virus minigenome system was developed by Palese and colleagues in 1989 [44], in which they modified influenza A virus by the addition of a reporter chloramphenicol acetyltransferase (CAT) gene, cloned between the 5′ and 3′ non-coding viral RNA segment sequences. The reporter gene construct was flanked by a promoter region for the T7 RNA polymerase and a restriction enzyme recognition site, which allowed for the formation of authentic viral 3′ ends. Following run-off, *in vitro* transcription of the viral-like RNA and the addition of purified polymerase and nucleoprotein, a reconstituted ribonucleoprotein (RNP) complex was produced. Subsequent transfection of the RNP complexes and infection with helper influenza virus was undertaken and a virus containing the virus-like RNA encoding CAT, in addition to the other influenza vRNAs, was generated. Although these experiments demonstrated the ability to generate modified infectious viruses, the particular system used requires a selection system to distinguish modified viruses from helper viruses [44].

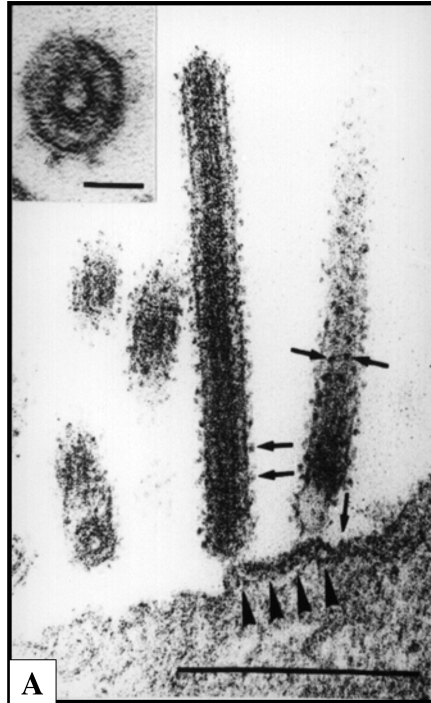
The generation of recombinant rabies virus by Conzelmann and colleagues in 1994 [70] demonstrated for the first time that generating a non-segmented negative-sense single-stranded (NNS) RNA virus entirely from a cDNA was possible. The cDNA encoding a full-length positive-sense copy of the rabies virus genome along with the nucleoprotein, phosphoprotein and RNA-dependant RNA polymerase, all under the control of the T7 RNA polymerase promoter, were transfected into eukaryotic cells. Infecting the transfected cells with recombinant vaccinia virus provided the T7 polymerase. This study helped to initiate the development of other genetic systems for members of *Mononegavirales*, including other members of *Rhabdoviridae* [10, 11, 39, 58, 63, 64] as well as *Paramyxoviridae* [2, 7, 8, 28, 33, 57, 61], which were based on T7 polymerase supplied by infection with ‘modified vaccinia virus Ankara’ (MVA-T7). In addition, more effective methods of providing the T7 RNA polymerase were also developed and included the use of plasmids expressing the protein [41, 52], expression of the polymerase from a stably transfected cell line [7, 61], and a heat shock method, which was shown to increase rescue efficiencies [57]. The utility of these alternative methods of supplying T7 were illustrated by the development of infectious clone systems for filoviruses. The first system developed for *Zaire ebolavirus* by Volchkov et al. in 2001 [78] was based on transfection of T7-driven plasmids encoding the genome as well as the nucleoprotein (NP), virion protein (VP) 35, VP30 and the RNA-dependent RNA polymerase (L) into BSR T7/5 cells, which stably expressed the T7 polymerase. Shortly thereafter, Neumann and

colleagues [52] demonstrated that T7 could be supplied via an additional plasmid, which encodes the T7 polymerase under the control of a eukaryotic promoter. An infectious clone system, also based on the transfection of BSR-T7 cells, has also been recently developed for borna disease virus. Further, using this system it has been demonstrated that, while cDNA constructs carrying the published genome sequence are functional, the rescued viruses are strongly attenuated, indicating that regulatory sequences of the viral genome determine virulence [69].

Infectious clone systems for segmented RNA viruses, although more complex, saw a breakthrough in 1996 when Bridgen and Elliott showed that the segmented Bunyamwera virus could be rescued using three anti-genome encoding plasmids in addition to viral protein expression plasmids [5]. This was the first rescue of a segmented negative-sense virus solely from cDNA. The establishment of minigenome and infectious clone systems for influenza A virus based on this technology followed these results, however, this system needed to be modified to deliver the vRNAs to the nucleus of transfected cells, where influenza A virus replication naturally occurs. This was overcome by the establishment of an RNA polymerase I based system [26, 54, 56]. In the case of the infectious clone, the optimized system required eight RNA polymerase I driven plasmids encoding the eight vRNA segments, in addition to four RNA polymerase II driven plasmids encoding the polymerase components (PA, PB1, and PB2) and the nucleoprotein [26, 54]. Hoffmann and Webster have modified the RNA polymerase system allowing both negative vRNA and positive-sense mRNA transcripts to be synthesized from the same template, thereby decreasing the number of plasmids required for influenza A virus rescue to eight [34]. Reverse genetics systems (minigenomes) have been published for other bunyaviruses such as Toscana and Rift Valley fever virus (genus *Phlebovirus*) [1, 43] and, more recently, Hantaan virus [23] and Crimean-Congo hemorrhagic fever (CCHF) virus [24], members of the genera *Hantavirus* and *Nairovirus*, respectively. While no infectious clone system has been developed to date for members of the family *Arenaviridae*, the recent development of a minigenome system for lymphocytic choriomengitis virus (LCMV) [40] and Tacaribe virus [42] is promising for the development of such a system in the near future.

Structure of filovirus particles

Filoviruses are enveloped, non-segmented, negative-sense (NNS) RNA viruses and constitute a separate family within the order *Mononegavirales*. The family consists of the genera *Marburgvirus* (MARV) and *Ebolavirus* (EBOV). The genus *Ebolavirus* is further subdivided into four distinct species: *Ivory Coast ebolavirus* (ICEBOV), *Reston ebolavirus* (REBOV), *Sudan ebolavirus* (SEBOV) and *Zaire ebolavirus* (ZEBOV) [18]. Filoviral particles are bacilliform in shape, but can also appear as branched, circular, U-shaped, 6-shaped, and long filamentous forms (Fig. 1A). They display a uniform diameter of approximately 80 nm, but vary greatly in length. Negatively contrasted particles, regardless of serotype or host cell, contain an electron-dense central axis (19–25 nm in diameter) surrounded by



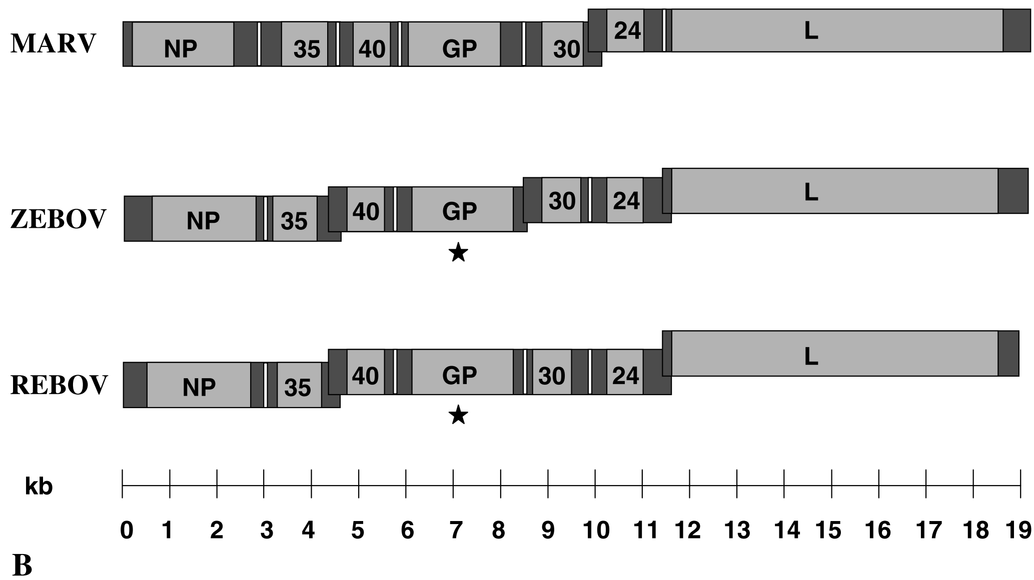


Fig. 1. Structure of filovirus particles. (**A**) Electron micrograph. Marburg virus (*MARV*) particles shown here demonstrate a characteristic bacilliform shape. The electron-dense central axis, formed by the ribonucleoprotein complex and the surrounding lipid envelope are clearly visible. Additionally, the glycoprotein (*GP*) can be observed as projections on the surface of the particles. [altered from reference #19] (**B**) Genome organization. The gene orders of fully sequenced filovirus genomes are presented. The intergenic regions are shown in white, the non-coding regions in dark gray boxes and the open reading frames in light gray boxes. Steps indicate the positions of the gene overlaps. Key: *REBOV Reston ebolavirus*; *ZEBOV Zaire ebolavirus*; *GP* glycoprotein; *kb* kilobase; *L* RNA-dependent RNA polymerase; *MARV Marburgvirus*; *NP* nucleoprotein; *24, 30, 35, 40* virion proteins (number indicates the molecular weight in kDa); * RNA editing site. [altered from reference #31]

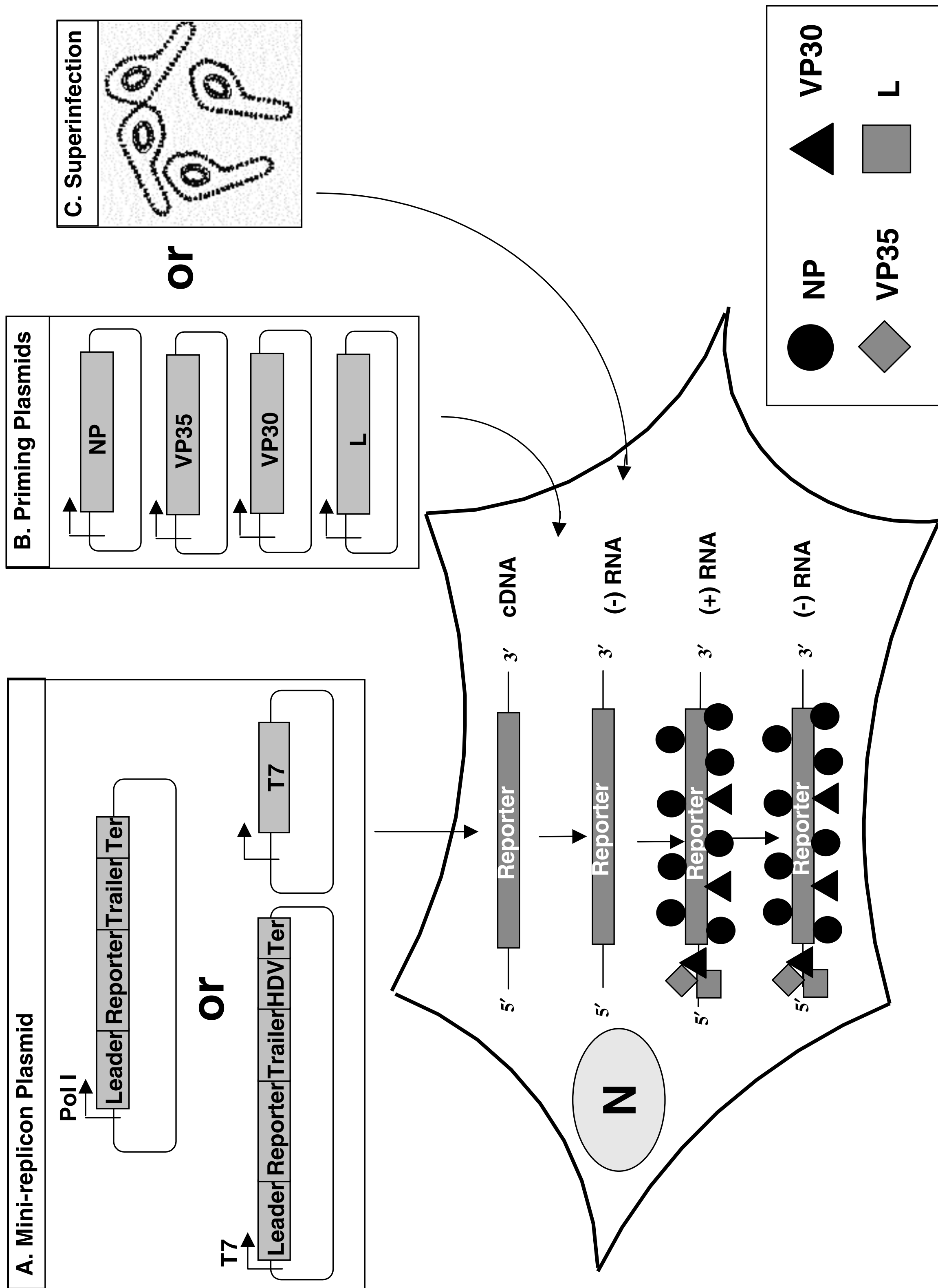
an outer helical layer (45–50 nm in diameter) with cross-striations at 5 nm intervals. This central core is formed by the RNP complex, which is surrounded by a lipid envelope derived from the host cell plasma membrane. Spikes of approximately 7 nm in diameter and spaced at about 5–10 nm intervals are seen as globular structures on the surface of virions (Fig. 1A) [29, 50, 60].

Virus particles are made up of seven structural proteins with presumed identical functions for the different viruses. Four proteins make up the RNP complex [nucleoprotein (NP), virion protein (VP) 35, VP30 and RNA-dependent RNA polymerase (L)] together with the viral RNA, while the remaining three proteins are membrane-associated [glycoprotein (GP), VP40, VP24]. The single type I transmembrane GP is inserted in the envelope as a homotrimer and functions in receptor binding and fusion; VP40 has been identified and characterized as the matrix protein but the function of VP24 is yet unknown. EBOV expresses a nonstructural soluble glycoprotein (sGP) as the primary gene product of the glycoprotein gene, which is efficiently secreted from infected cells; its functions remain unknown [19, 21, 49, 53, 67, 77, 85].

The single negative-sense linear RNA genome of filoviruses does not contain a poly(A) tail and is noninfectious. Upon entry into the cytoplasm of host cells it is transcribed by the viral polymerase to generate polyadenylated subgenomic mRNA species. Filovirus genomes are approximately 19 kb in length and genes are organized in the following linear order: *3′ leader – NP – VP35 – VP40 – GP – VP30 – VP24 – L - 5′ trailer* (Fig. 1B). Genes are delineated by transcriptional signals at their 3′ and 5′ ends that have been identified by their conservation and by sequence analysis of mRNA species. The sequences *3′-CUNCNUNUAAUU-5′* and *3′-UNAUUCUUUUU-5′* represent the consensus motifs for transcriptional start and stop signals, respectively. Filoviral genes are usually separated from each other by intergenic regions that vary in length and nucleotide composition, but some gene overlaps occur at characteristic positions (Fig. 1B). The length of the overlaps is limited to five highly conserved nucleotides within the transcriptional signals (3′-*UAAUU*-5′) that are found at the internal ends of the conserved sequences. Most genes tend to possess long non-coding sequences at their 3′ and/or 5′ ends, which contribute to the increased length of the genome. Extragenic sequences are found at the 3′-leader and 5′-trailer ends of the genome. The leader and trailer sequences are complementary to each other at the extreme ends; a feature that is shared by many NNS RNA viruses [19, 49, 67].

Minigenome systems for filoviruses

In the case of filoviruses, minigenome systems for MARV (strain Musoke) and ZEBOV (strain Mayinga) were developed using the T7 RNA polymerase to synthesize negative-sense vRNA transcripts from cDNA [47, 48]. Initially, the cDNA constructs contained the 3′ leader, the non-coding region of the NP gene, the 5′ non-coding region of the L gene and the 5′ trailer sequences of the genome flanking the single reporter gene CAT (Fig. 2). These minigenome systems were driven either by a helper virus infection or transfection of plasmid DNA encoding


A. Mini-replicon Plasmid
Pol I
Leader
Reporter
Trailer
Ter
or
T7
Leader
Reporter
Trailer
HDV
Ter
T7
B. Priming Plasmids
NP
VP35
VP30
L
or
C. Superinfection
cDNA
(-) RNA
(+) RNA
(-) RNA
5′
3′
Reporter
N
NP
VP35
VP30
L

the RNP complex proteins to provide the necessary machinery for transcription and replication. In the past 5 years, both systems have allowed the study of different aspects of filovirus transcription and replication.

Using the minigenome systems it was established that only three of the four-nucleocapsid proteins, NP, VP35 and L, were necessary to support replication and transcription of the monocistronic MARV minigenomes [47]. This is in agreement with data obtained from various paramyxovirus and rhabdovirus systems where the nucleoprotein (N), phosphoprotein (P) and the RNA-dependent RNA polymerase (L) are the minimum proteins required for transcription and replication [12, 16, 32, 37, 61, 71, 88]. Minigenome systems developed for members of the family *Bunyaviridae* (tri-segmented negative-sense RNA viruses) were transcribed and replicated using only the NP and L protein [15, 23–25, 43].

The minigenome system for ZEBOV, which was subsequently generated using the same strategy as for the MARV system, required all four of the nucleocapsid proteins NP, VP35, VP30 and L for efficient replication and transcription of the monocistronic minigenomes [48]. Utilizing the plasmid based minigenome system it was further shown that VP30 of ZEBOV could efficiently enhance transcription of the ZEBOV minigenome [48]. The presence of VP30 might resolve or cover RNA secondary structures either by RNA binding or by directing an additional co-factor to the folding RNA. However, thus far, RNA binding activity for VP30 has not been described, and since the ZEBOV genome is bound by NP, secondary structure formation may not occur. The only naked RNA species present are the positive-sense mRNA transcripts, which have been shown to form secondary structures. Thus, it could be possible for VP30 to have an effect on these secondary structures at the mRNA level [84]. Our knowledge of VP30 was extended when VP30 was found to contain two N-terminal serine clusters, which positively

Fig. 2. Transcription and replication steps in a minigenome system. To examine replication and transcription in a minigenome system, cells have to be transfected with a minigenome plasmid (**A**) that contains a reporter gene flanked by the genomic leader and trailer regions under control of either a T7 or a Pol I promoter. The T7-driven minigenome contains an additional hepatitis delta virus (HDV) ribozyme sequence, which results in transcript cleavage to generate an authentic genome end. In the case the T7-driven constructs, T7 polymerase has to be introduced into the cells either by transfecting a plasmid coding for it, infection with a recombinantly T7 expressing vaccinia virus, or by using cell lines that express this protein constitutively. Once generated further transcription and replication of viral like RNA species can be driven either by helper plasmid encoded ribonucleoprotein (*RNP*) complex components [nucleoprotein (*NP*), virion protein (*VP*) 35, VP30, RNA-dependent RNA polymerase (*L*)] (**B**) or by infection with live virus (helper virus) (**C**). The transfected cDNA is transcribed into a virus-like vRNA in negative orientation by either the T7 or the Pol I polymerase. Subsequently, this vRNA is replicated by the proteins of the viral RNP complex into cRNAs in positive and vRNAs in negative orientation. Transcription of reporter mRNA transcripts from the vRNA-like minigenomes by the RNP complex proteins and subsequent translation leads to expression of the reporter gene. *N* nucleus; *Pol I* RNA polymerase I; *T7* bacteriophage T7 RNA polymerase; *Ter* terminator

regulated the binding of VP30 to NP, and in doing so negatively regulated the transcription activation function of VP30. It was also shown that VP30 is a target for the cellular protein phosphatases PP1 and PP2A. In a reconstituted minigenome system, ZEBOV specific transcripts were blocked by okadaic acid, which is known to inhibit PPI and PP2A. Treatment of ZEBOV infected cells with okadaic acid also inhibited ZEBOV growth, which could be compensated for by the expression of a non-phosphorylated VP30 *in trans* [45]. All these results taken together illustrate that VP30 phosphorylation is a regulatory factor in the replication cycle of ZEBOV.

A literature search revealed that several attempts have been made to investigate whether non-segmented negative-sense, single strand RNA virus replication complexes were able to recognize heterologous RNA templates *in vivo*. It was reported that human parainfluenza virus (HPIV) type 1 and type 3 could accept a Sendai virus minigenome as a template for replication, whereas measles virus could not. The same rescue results were also seen when others utilized a plasmid-based artificial replication system [59]. However, the rescue of the HPIV type 3 minigenome could not be supported by respiratory syncytial virus (RSV) or, unexpectedly, by bovine parainfluenza virus type 3 [13]. For Toscana and Rift Valley fever virus (genus *Phlebovirus*, family *Bunyaviridae*) it was demonstrated that the transcription complexes were active on heterologous template [1], and for vesicular stomatitis viruses (VSV) it was shown that replication of defective interfering particle RNAs from serotypes New Jersey and Indiana was possible but only when the replication complex was supplied by VSV Indiana [46]. These data illustrate that the specificity of the replicase complex to the target sequences is not absolute and depends on the virus system. In general, MARV/ZEBOV minigenome systems supported transcription/replication of homologous but not heterologous RNA templates, regardless whether helper virus or transfected nucleocapsid complex protein expression plasmids were used for transcription/replication of the minigenome system. As an exception, MARV VP30 could replace ZEBOV VP30 in the ZEBOV minigenome system, although this switch resulted in lower activity than the native ZEBOV VP30 protein [48]. However, a chimeric minigenome system containing the ZEBOV leader and the MARV trailer was shown to be encapsidated, replicated, transcribed, and packaged by both viruses [48].

Recently, our group has developed a minigenome system for REBOV [30] employing an alternative to the classical T7-driven approach used with MARV and ZEBOV. This system was based on an initial transcription step mediated by RNA polymerase (Pol) I, an endogenous host polymerase. Thus, minigenome transcription by Pol I eliminates the need to introduce a source of the polymerase into mammalian cells (see Table 1 for a comparison of the respective polymerase properties). While introduction of T7 into mammalian cells can be achieved in a number of ways, the need to do so presents a potential limitation if the entire population of cells is not targeted. Minigenome transcription by Pol I also overcomes a number of limitations of the T7-driven system relating to the

Table 1. Comparison of polymerase properties

	T7 RNA polymerase	RNA polymerase I
Origin	bacteriophage	eukaryotic
Methods of introduction	MVA-T7 infection, transient or stable transfection	N/A
Localization	cytoplasmic	nuclear
mRNA Modification	5′ capping and 3′ polyA*	no
Initiation/termination	additional 5′ and 3′ nucleotides	no additional nucleotides

*Occurs only if using MVA-T7 infection as source of T7 RNA polymerase
MVA-T7 Modified vaccinia virus Ankara recombinantly expressing the bacteriophage T7 polymerase

production of authentic, non-modified transcripts which have correct sequences at their termini (Table 1). Despite the exclusively cytoplasmic replication of filoviruses, the nucleolar localization of Pol I within host cells does not appear to present a barrier to successful rescue of high levels of reporter activity from this system, as has been shown previously with several bunyavirus systems [23–25]. On the contrary, our data indicate that a Pol I-driven REBOV minigenome generates both a higher level of reporter activity and a higher signal-to-noise ratio than did a comparable T7-driven REBOV construct and, thus, this system seems to help overcome some of the technical limitations of T7-mediated transcription [30]. The successful establishment of a REBOV minigenome system is of particular interest as a tool to study transcription and replication of this virus in comparison to more pathogenic filoviruses.

Infectious clone systems for filoviruses

As noted earlier, minigenome systems are the building blocks of an infectious clone. These systems utilize the same principles as a minigenome system, but rather than a reporter gene being expressed by the nucleocapsid proteins, the full viral genome is replicated and transcribed, producing live infectious, fully functional viruses (Fig. 3). Two such reverse genetic systems have been developed for ZEBOV. The first system developed by Volchkov and colleagues utilized a cell line, which stably expressed the T7 polymerase (BSR T7/5) [78]. The T7 polymerase drives the transcription of the cDNA copy of ZEBOV, producing a negative-sense RNA molecule. This RNA species can be used for the replication of an antigenomic template (positive-sense RNA) producing both RNA species used in viral transcription and replication. In contrast to this system, Neumann and colleagues [52] developed a ZEBOV reverse genetic system, which successfully utilized a plasmid driven T7-RNA polymerase rather than the BSR T7/5 cell line (Fig. 3). These two methods are both sufficient to provide the T7 RNA polymerase. For the development of previous systems, the T7 RNA polymerase

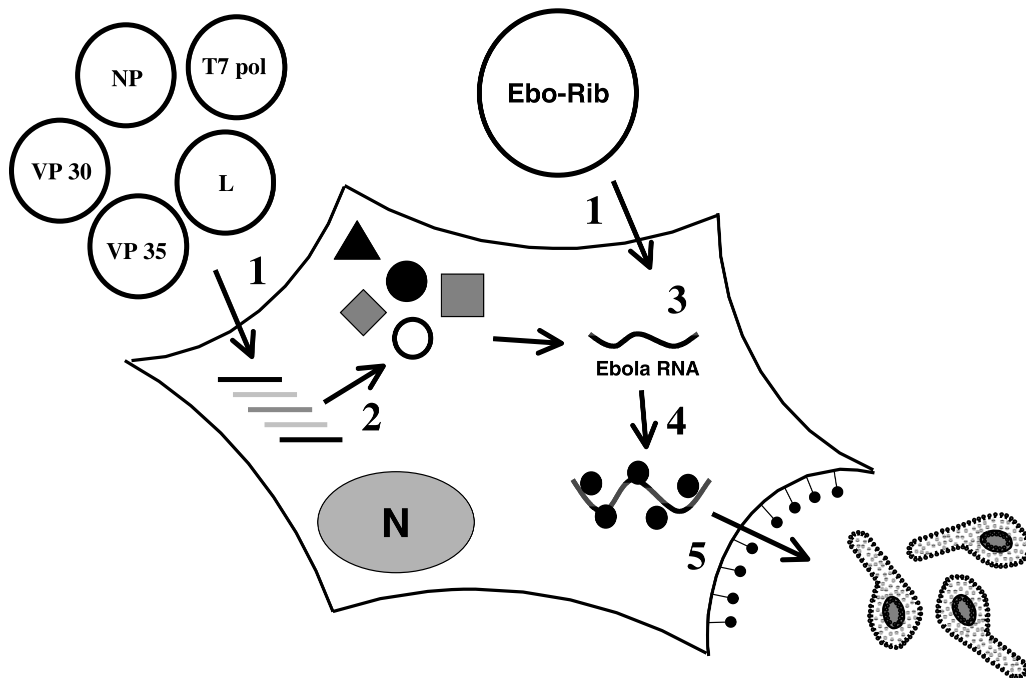


Fig. 3. Zaire ebolavirus (*ZEBOV*) infectious clone system. The scheme illustrates the components of the system and the steps involved in the rescue of infectious virus. (*1*) Co-transfection of the plasmid carrying the full-length ZEBOV genome and the expression plasmids for the bacteriophage T7 RNA polymerase (*T7 Pol*) and the four ZEBOV proteins associated with the ribonucleoprotein complex (*L, NP, VP30, VP35*); (*2*) – expression of the viral support proteins and the bacteriophage T7 RNA polymerase under the control of the chicken β-actin promoter; (*3*) – transcription of the ZEBOV genome under the control of the bacteriophage T7 RNA polymerase promoter; (*4*) – formation of the ribonucleoprotein complex, transcription and replication; (*5*) – virus maturation at the plasma membrane and subsequent budding of infectious virus particles. *L* RNA-dependent RNA polymerase; *N* nucleus; *NP* nucleoprotein, *VP* virion protein 30 and 35 kDa. [altered from references #52 & #74]. ● NP; ◆ VP35; ○ T7; ▲ VP30; ■ L

has been commonly provided by infection with a recombinant vaccinia virus [51, 55]. However, this system has the disadvantage of requiring separation of the recombinant viruses of interest from progeny of the recombinant vaccinia virus. Recently we have optimized the infectious clone system developed by Neumann and colleagues [52] to a rescuability of nearly 100% [74]. This system can now be more reliably used for the generation and analysis of mutants, particularly if rescues are unsuccessful due to incompatibility of the mutations with virus replication.

The ZEBOV infectious clone systems have been used in the past to address questions regarding the pathogenic potential of GP, which is encoded by gene 4 of the linear arranged genome (Fig. 4). In contrast to MARV, which produces only $GP_{1,2}$, the predominant products of this gene for all EBOV species are the soluble secreted glycoproteins sGP and Δ-peptide, a small carboxyl-terminal

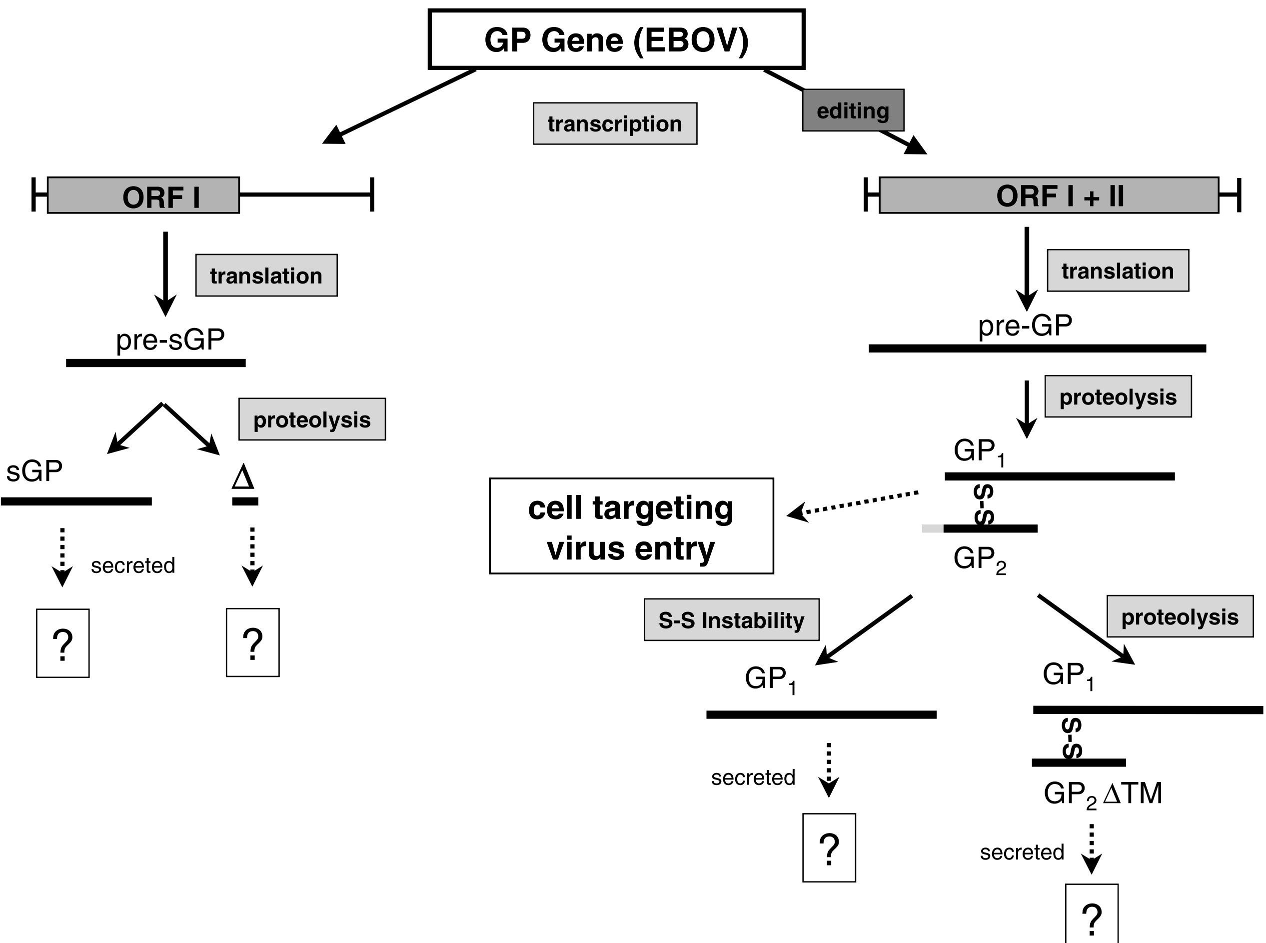


Fig. 4. Ebola virus glycoprotein processing. Through a process of transcriptional editing at a series of 7 adenosine residues EBOV shifts its open reading frame and creates an mRNA transcript encoding the precursor of the full-length glycoprotein, pre-GP. This precursor protein is then proteolytically cleaved by furin or furin-like endoprotease into GP_1 and GP_2, which are linked by a disulfide bridge and expressed on the surface of the cell as $GP_{1,2}$. Destabilization of the disulfide bridge leads to release of a soluble form of GP_1. In addition, metalloprotease cleavage produces another soluble form of the glycoprotein, $GP_{1,2\Delta TM}$. Without editing, a precursor of a soluble form of the glycoprotein, pre-sGP, is produced and subsequently cleaved by furin or a furin-like endoprotease into two secreted products sGP and Δ peptide. The function of the secreted protein species is currently unknown, however, the membrane bound full-length $GP_{1,2}$ mediates cell targeting and virus entry. *EBOV* Ebola virus; *ORF* open reading frame; *pre-sGP* precursor from which sGP and Δ peptide are produced by proteolytic cleavage; *pre-GP* precursor from which GP_1 and GP_2 are produced by proteolytic cleavage. Note, MARV does not use RNA editing for the expression of preGP and, thus, does not express the soluble glycoproteins sGP and Δ-peptide. [altered from reference #21]

peptide generated through cleavage by furin or a furin-like endoprotease from a precursor (pre-sGP) [81, 82]. The GP, found on the surface of mature EBOV particles, is produced through transcriptional RNA editing [66, 75], while that produced by MARV results from direct transcription and translation of the single open reading frame (ORF). $GP_{1,2}$ facilitates receptor binding and fusion with target cells and is associated with host cell cytotoxicity [27, 35, 36, 65, 72, 83, 87]. GP is proteolytically processed by furin or a furin-like endoprotease into the cleavage fragments GP_1 and GP_2, which are disulfide linked and form the mature spike protein [68, 76]. During processing, $GP_{1,2}$ becomes partially instable and the non-membrane-bound fragment GP_1 gets released from infected cells [79]. Recently, it was shown that another soluble product, $GP_{1,2\Delta TM}$, is produced through metalloprotease cleavage of the membrane-bound mature $GP_{1,2}$ [14]. The mature $GP_{1,2}$ is known to form homotrimers on the surface of particles and it is speculated that trimerization is mediated through the GP_2 component of the protein [22, 68]. The expression strategy of the glycoprotein gene and the roles of the different expression products have been summarized in detail in several review articles [21, 77].

Volchkov and colleagues [78] investigated the importance of the editing site within the glycoprotein of EBOV. The editing site, which consists of seven consecutive adenosine residues, is located within the GP gene at nucleotide positions 6918–6924 (AF272001). Interestingly, about 80% of the glycoprotein gene-derived mRNA transcripts in infected cells are not edited, but can direct the synthesis of the nonstructural glycoproteins sGP and Δ-peptide [66, 75]. Both proteins are secreted from EBOV-infected cells and sGP has also been detected in blood of EBOV infected patients [66]. Using site directed mutagenesis, the seven-adenosine residues were interrupted by adding two guanidines (AAGAAGAA) and an additional adenosine to keep the defined open reading frame, such that only $GP_{1,2}$ is produced [78]. This construct was rescued and the effects of an editing-deficient virus were assayed. It was demonstrated that without editing, effective replication and transcription were unhindered. However, the increased expression of full-length GP did not simultaneously increase viral release as one might have expected, but resulted in a stronger cytopathic effect. It was demonstrated that GP synthesis was of an immature precursor with high-mannose type sugars, indicating that glycoprotein transport was largely arrested in the endoplasmic reticulum or in an early Golgi compartment [78]. Thus, overexpression of the GP might lead to cell death by exhausting the processing machinery of the cells. On the other hand, there is evidence that $GP_{1,2}$ displays cytotoxicity by itself which seems to be associated with the transmembrane subunit GP_2 and/or the mucin-like domain found in GP_1. However, cytotoxicity depends on the level of glycoprotein expression and, thus, expression of sGP (non-edited transcript) seems to control the cytotoxicity associated with the transmembrane $GP_{1,2}$, leading to enhanced virus load and spread in the infected host [21].

Neumann and colleagues [52] have studied the importance of the proteolytic processing of the transmembrane glycoprotein precursor (pre-GP) for infectivity

of virions. As mentioned above, it had been shown previously that EBOV transmembrane GP is cleaved by a subtilisin-like endoprotease such as furin [76]. Interestingly, studies with murine leukemia virus [86] and VSV [36] pseudotyped with mutant ZEBOV GP lacking a furin recognition site indicated that glycoprotein cleavage was not necessary for infectivity of the pseudotyped viruses. However, for many viruses, posttranslational cleavage of membrane glycoproteins by host proteolytic enzymes, including subtilisin-like proteases such as furin, is a prerequisite for fusion between the viral envelope and cellular membranes and, therefore, is an important step in pathogenesis [38]. For viruses in the families *Orthomyxoviridae* and *Paramyxoviridae*, glycoprotein cleavage by furin and other host cell proteases is required for their infectivity and thus determines the extent of viral pathogenicity [38]. As previously mentioned, MARV and EBOV are proteolytically processed by furin or furin-like proteases at a highly conserved sequence (R-X-K/R-R; X, any amino acid) [36, 76, 80]. Since the glycoprotein amino acid sequence of REBOV, the least pathogenic of all EBOV species in humans, deviates from the optimal furin recognition sequence (Fig. 5A), GP cleavage has been thought to be an important determinant of filovirus pathogenicity [21]. Using the infectious clone technique, Neumann and colleagues [52] destroyed the furin recognition motif at the predicted cleavage site of ZEBOV by site directed mutagenesis. The resultant rescue of ZEBOV with uncleaved GP indicated that cleavage is not necessary for *in vitro* replication. The GP mutant virus was slightly attenuated *in vivo* showing decreased titers in tissue culture growth, indicating that cleavage may enhance infectivity. The EBOV fusion peptide has an unusual location 28 amino acids from the amino-terminal end of GP_2 [35, 68] (Fig. 5B and 5C). It is also flanked by two cysteine residues which are thought to form a disulfide bridge, thus, exposing the fusion peptide in form of a loop [21, 27]. This unusual localization and structure might allow sufficient exposure of the fusion peptide to interact with the cellular membrane, even if GP remains uncleaved and thus, could explain that infectivity is largely independent of cleavage (Fig. 5B and 5C). However, the appearance and secretion of GP_1 (Fig. 4) is dependent on cleavage of preGP, and cleavage might, therefore, be essential for pathogenicity since soluble GP_1 has been postulated as a pathogenic determinant for filoviruses [20, 21].

Similar to other non-segmented negative-sense RNA viruses and data obtained with the filovirus minigenome systems [47, 48], transcription and replication of EBOV are thought to be highly specific processes depending on the interaction of the viral RNA polymerase and its co-factors with homologous template RNA. However, using the infectious clone technology it was determined that these processes are less restrictive than expected [74]. The functional specificity of the different viral proteins associated with the ribonucleoprotein complex could be substituted with heterologous support proteins derived from closely and more distantly related filoviruses, such as REBOV and MARV, respectively [74]. These data clearly demonstrate that transcription and replication are not strictly species-specific but require a certain degree of specificity, as indicted by a lower degree of rescuability when heterologous support proteins were derived from evolutionarily more distant viruses. The studies further indicate that protein–protein interactions

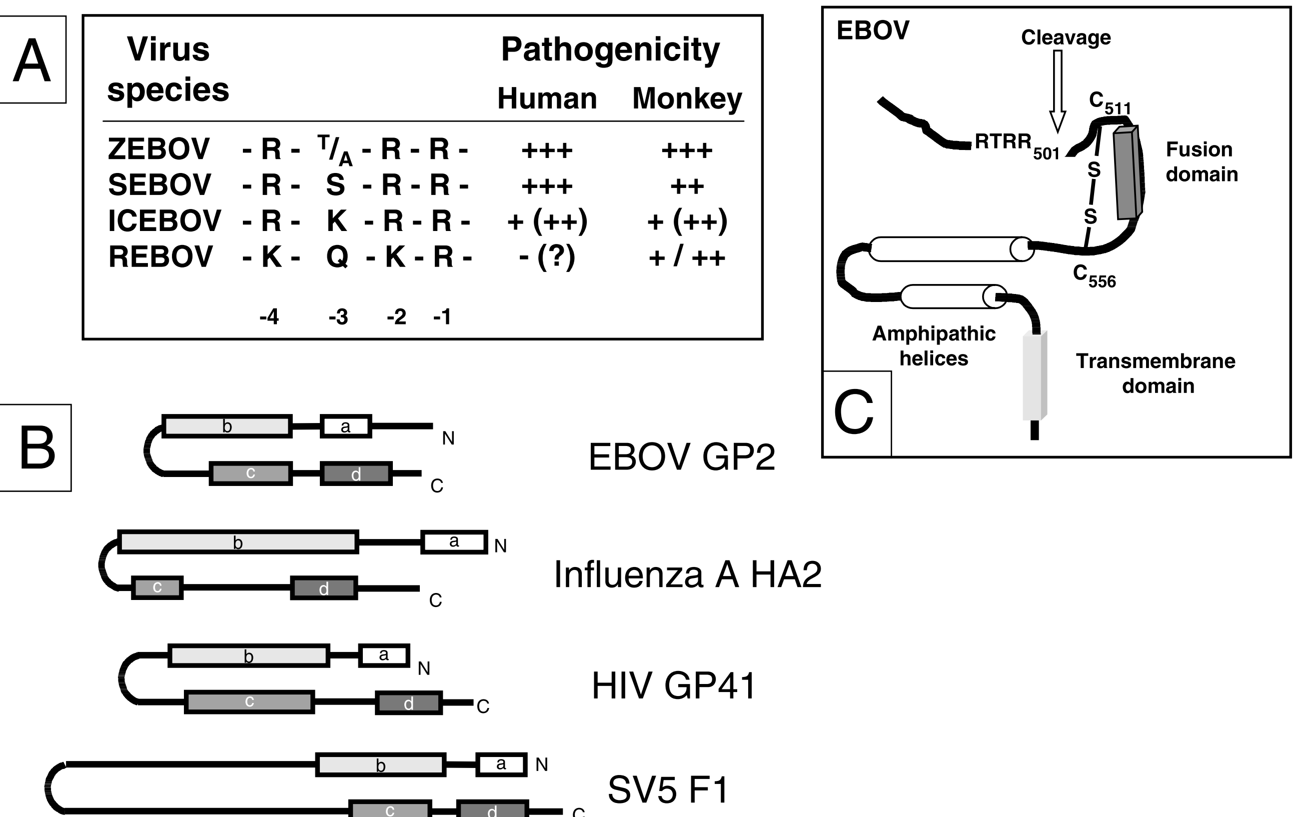


Fig. 5. Structure of Ebola virus GP_2 (**A**) Proteolytic cleavage sites of filovirus glycoproteins. The amino acid sequences of the cleavage sites are presented from positions −1 to −4. Proteolytic cleavage occurs carboxy-terminal to the arginine residue at position −1. The relative pathogenicity in human and non-human primates is indicated. (**B**) Structural features of fusogenic transmembrane glycoprotein domains. Structural similarities between EBOV GP_2 and the transmembrane subunits HA2 of the influenza virus hemagglutinin, gp41 of the HIV env protein and F1 of the SV5 virus fusion protein are shown. Four domains can be distinguished in the fusion active state: the fusion peptide (*a*), an amino-terminal helix (*b*), a carboxy-terminal helix (*c*) and the membrane anchor (*d*). The transmembrane proteins assemble into trimers in which the large amino-terminal helices form an interior, parallel coiled-coil, while the smaller carboxy-terminal helices pack in an anti-parallel fashion at the surface. Therefore, the fusion peptide and the membrane anchor are located at one end of the rod-like trimers. (**C**) Proposed structure of GP_2. The ectodomain of GP_2 contains the fusion peptide followed by an amino-terminal helix, a peptide loop and a carboxy-terminal helix. Helices were proposed by the GARNIER program of PC/GENE (IntelliGenetics Inc.). The fusion peptide is predicted to be exposed on a loop formed by the disulphide linking of cysteine residues 511 and 556. [altered from reference #21]

within the ribonucleoprotein complex might be more critical than protein-RNA interactions. As demonstrated before using the minigenome system [48], the presence of an EBOV-derived VP30 was critical for the rescue of ZEBOV [74].

Past and future challenges

Major problems with infectious clone systems relate to the handling of the larger plasmids, such as the genomic plasmid and the plasmid encoding the L protein. One common occurrence is spontaneous mutations, which have occurred in the generation process of the two existing systems. The first system [78] carried a single mutation in the genomic clone at nucleotide position 18227 (within the L-gene) which can be attributed to a polymerase error during RT-PCR in the original development. This mutation was silent and did not have a recognizable effect on viral rescue or viral transcription or replication. The cDNA clone that was developed, by Neumann and colleagues [52] showed three nucleotide changes. The first mutation was an A insertion between nucleotide positions 9744 and 9745. Another A insertion was found between nucleotides 18495 and 18496 and an A-to-T replacement was detected at position 18226. Interestingly, all of the mutations found in the cDNA full-length clone had been reported to be present in the functional ZEBOV minigenome [48] or have been found with other ZEBOV strains (see data bank sequences) and are, therefore, considered naturally occurring variants. Neither the insertions nor the replacement mutations had an effect on the virus once rescued, indicating that some minor mutations seem to be tolerated within the cDNA full length constructs without effecting rescuability. Volchkov and colleagues [78] took advantage of this by intentionally inserting a silent mutation at nucleotide position 6180 to create a unique SalI restriction enzyme site, which subsequently was used to identify rescued mutant viruses from wild-type ZEBOV.

These mutational problems not only occur when developing a cDNA viral genome copy but have been a recurring event when cassette mutagenesis and full length cDNA re-cloning are carried out. To alleviate this problem a new cDNA full-length reverse genetic clone has been established for ZEBOV, which encodes a pBR322 origin [17]. This change decreased the plasmid copy number and has, thus, decreased the probability of a spontaneous mutation occurring when mutagenizing cDNA fragments. Another method, which has been used in mutatgenesis strategies when working with these large plasmids, is developing smaller cassettes of the cDNA plasmid. These cassettes can then be easily mutagenized and re-cloned into the full-length cDNA viral plasmid allowing for easier cloning and development of mutant viruses.

In conclusion, reverse genetics for filoviruses are likely to become extremely valuable research tools in future. The existing minigenome systems for ZEBOV [48], REBOV [30], and MARV [47] are first choices for deciphering the mechanisms of viral replication and transcription, work that has already made great progress since the development of these systems (see above). In addition, they will be helpful for screening antiviral drugs targeting the replicase complex of

filoviruses, a priority for the response capacity against A List bioterrorism agents, such as EBOV and MARV [3, 6]. The infectious clone systems, currently only existing for ZEBOV (wild-type) [52, 78] will become key elements for pathogenesis studies and might be helpful for vaccine development. Pathogenesis studies are dependent on animal models. Since the rodent models for filoviruses are dependent on adapted virus strains, it will be important to develop infectious clone systems for the mouse-adapted ZEBOV [4], and the guinea pig-adapted ZEBOV [9] such systems are currently under development [17].

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Structural biology of old world and new world alphaviruses

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Introduction

Alphaviruses (family *Togaviridae*, genus *Alphavirus*) have been classified as belonging to either Old World or New World types depending on where they occur [4]. Some Old World alphaviruses such as Sindbis virus are found in Africa, Europe, Asia and Australia, whereas New World alphaviruses such as Venezuelan equine encephalomyelitis virus (VEEV) are found in the Americas. Alphaviruses are typically transmitted to humans and animals through the bite of an infected mosquito. In general, Old World alphaviruses cause less severe disease and have lower mortality rates in humans relative to New World alphaviruses [7]. Old World alphavirus infections are typically characterized by rash and arthritis, while New World alphavirus infections are typically characterized by debilitating febrile disease and, sometimes, encephalitis. Because of their disease severity, and their ease of growth and transmission, many New World alphaviruses are classified as category B bioagents. New World alphaviruses can also cause severe economic impacts, since they cause disease in livestock as well as in humans.

Alphaviruses are positive-sense ssRNA enveloped viruses that measure ~700 Å in diameter. They are composed of two membrane associated glycoproteins, a host cell derived lipid bilayer, and a nucleocapsid composed of capsid proteins and a 49S RNA molecule. The outer envelope is believed to be made up of 80 E1/E2 heterotrimers arranged as 120 dimers of the E1 protein around 80 homotrimers of E2 in a T = 4 icosahedral lattice [7, 8, 13]. The 400 Å diameter nucleocapsid is made up of 240 copies of the capsid protein organized in a T = 4 icosahedral arrangement [11]. The envelope proteins and the nucleocapsid are believed to interact through C-terminal residues of E2 that are exposed on the inner surface of the lipid bilayer and the capsid protein C-terminal domain. The

E1–E1 glycoprotein interactions form the scaffolding lattice, which help stabilize the virus icosahedral structure. The E2 glycoprotein is the primary component of the morphological spikes and is responsible for host cell receptor recognition [1].

The mechanism by which alphaviruses penetrate host cells has been studied for many years. It has been widely accepted that many enveloped viruses employ a fusion-type mechanism to pass through the host cell membrane and initiate infection [16, 17]. This conclusion was based in part on early observations that cell-to-cell fusion of infected cells occurred upon exposure of the virus to low pH [14]. Although many enveloped viruses do not rely on low pH to trigger fusion, for those that do the endosome was believed to be the site of virus-host cell penetration because it was the only site within the cell where the virus could be expected to encounter a low pH environment. A notable exception to the endosomal route in a system shown to demonstrate low pH triggered fusion, was the alphaviruses. Although alphavirus-mediated fusion has been observed, this phenomenon has not been demonstrated to occur at the endosomal pH. Rather, for alphavirus-mediated fusion to be observed, alphaviruses attached to cells must be briefly exposed to low pH, and returned to neutral pH for fusion to occur [3]. This is a situation never expected to occur within an endosome. Alphavirus penetration may occur instead through the action of a pore formed from the interaction between the virus and the host-cell receptor. It is through this pore that virus RNA may be injected into the cell cytoplasm [12].

Structural studies of alphaviruses and components

Because of their impact on human health and the livestock industry, alphaviruses are an important group of viruses to study. Their RNA and protein sequences are highly conserved among species, suggesting that their three-dimensional structures may be similar [4]. The low resolution structures of Sindbis, Semliki Forest (SFV), Ross River, VEEV and Aura viruses support this generalization. However, since alphavirus lineages display significant differences in tissue tropism and pathobiology [7], it is possible that local conformational differences between the viruses and their molecular components are responsible for their different phenotypes.

High-resolution structural studies of alphaviruses are complicated by their enveloped nature and their pathogenicities. Three-dimensional alphavirus crystals, although relatively easy to produce, typically have not diffracted beyond 30 Å resolution [5, 18]. X-ray structural studies have been limited to the individual viral proteins [2, 8]. While the structures of stable domains of the E1 and capsid protein have been determined by X-ray crystallography, the structure of E2 is unknown. The difficulty in producing diffraction-quality E2 crystals may lie in the strong affinity between E2 and E1.

Isolated alphaviruses are icosahedral, highly homogenous, and stable. These properties facilitate electron cryomicroscopy (cryo-EM) structural studies of these viruses. Cryo-EM has succeeded in generating intermediate resolution structures of SFV, Sindbis virus and VEEV [10, 11, 20]. Combining high-resolution X-ray

structures with cryo-EM structures has allowed us to generate pseudo-atomic models of the virus and its intermolecular interactions. This approach has been used to investigate intermolecular contacts among and between the nucleocapsid and E1 proteins [8, 20].

Structural studies of VEEV

VEEV is one of the more virulent alphaviruses and poses a significant health threat within the Americas [15]. Understanding its structure is vital for developing effective strategies to combat VEEV infections. The structure of TC-83 VEEV has been determined by cryo-EM to 15 Å resolution (Figs. 1–3). The 680 Å diameter structure is composed of 80 E2 trimers arranged on a $T = 4$ lattice. This envelope structure appears similar to the 11 Å resolution structure of SFV [10]. Both structures show that the trimers are flattened at the extended tips, possibly to display the receptor recognition site on the virus surface. In VEEV virus, the trimers rise ∼84 Å above the outer leaflet of the virus membrane (Fig. 3). As was described for SFV, the outer spike layer is divided into two regions, the exposed projecting domains and the skirt region [10]. In our reconstruction, the projecting domains measure 48 Å and sit on top of the skirt region which is 36 Å deep. There is evidence that the projecting domains are primarily E2 while the skirt region is composed of E1 [1, 8]. Interactions between the E2 proteins in the center of the timer seal the cavity within the skirt region in both SFV and VEEV [10].

The cryo-EM structures from different alphaviruses are sufficiently resolved to detect subtle structural differences. For instance, the 400 Å diameter nucleocapsid of VEEV (Fig. 2) is structurally different from those of the Old World alpha-

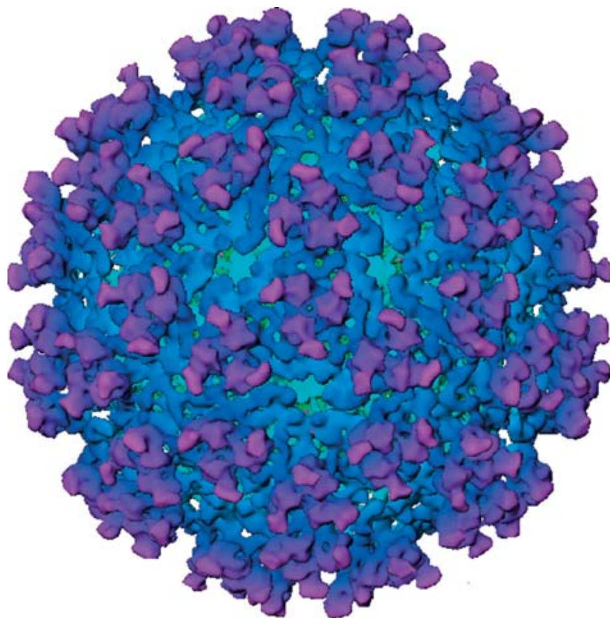

Fig. 1. 15 Å structure of Venezuelan equine encephalomyelitis virus (680 Å in diameter) generated from approximately 900 particle images recorded in a JEOL4000 electron cryomicroscope operated at 400 kV and −170 °C specimen temperature. The map has been bilaterally filtered [6]. The surface representation of the map is colored according to radius

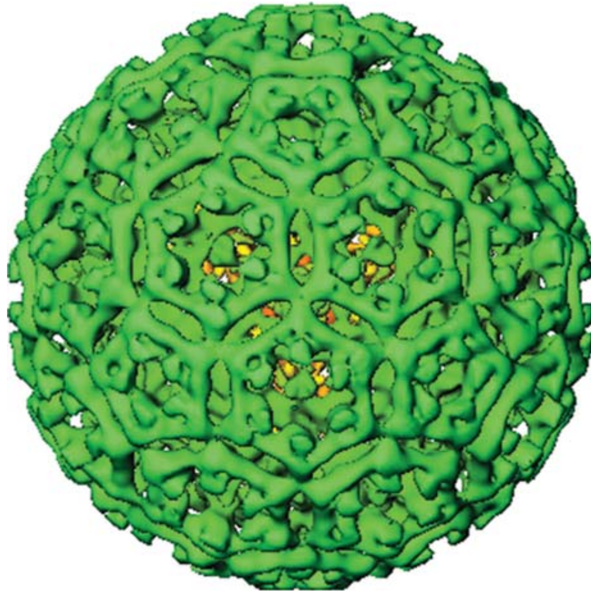

Fig. 2. VEEV Nucleocapsid (∼400 Å in diameter) viewed along the 3-fold axis

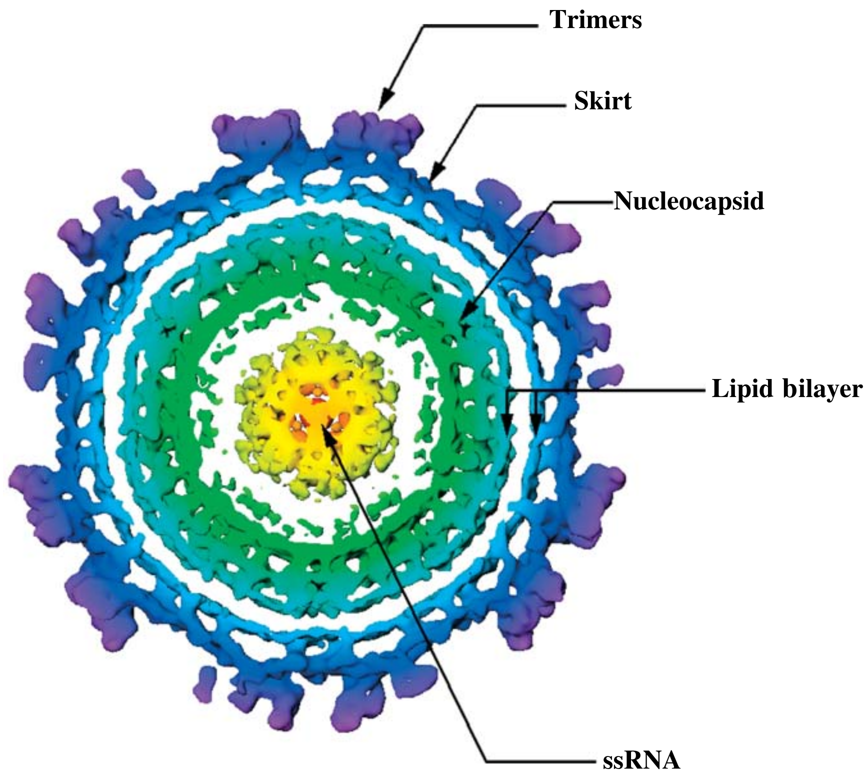

Fig. 3. Slice (53 Å in thickness) of density of the VEEV normal to the 3-fold axis. The slice shows the outer most trimers which are composed primarily of E2 and sit on the skirt region. The skirt is composed of E1–E1 interactions forming the icosahedral structure of the virus through which the transmembranal domains of E2 pass to associate and bind to the virus nucleocapsid. The slice also includes regions attributed to the nucleocapsid, the lipid bilayer and the ssRNA

viruses. In all alphavirus structures determined so far, the 240 individual capsid proteins are arranged into 12 pentamers and 30 hexamers in a $T = 4$ arrangement. In structures of Old World alphaviruses, the nucleocapsids appear to have a slight clockwise twist of the pentamer relative to the hexamer. This twist of nucleocapsid pentamers and hexamers is not seen in the VEEV nucleocapsid, providing the first evidence that the structures of New and Old World alphaviruses differ. In VEEV, the vertices of each pentamer point towards the two fold axis of symmetry at the vertices of two neighboring hexamers, forming a strong local three fold axis of symmetry (Fig. 2). A reexamination of the New World Aura virus has shown that its nucleocapsid is arranged similar to that of VEEV [19]. This difference in nucleocapsid structure can be traced to the different capsid–capsid interactions that likely exist within the VEEV nucleocapsid. Based on examination of 15 Å resolution structures, these differences do not appear to be transmitted through the viral membrane to the envelope proteins because the VEEV envelope appears to adopt a conformation that is structurally similar to that of the Old World alphaviruses. Given the observation that New World alphaviruses typically cause a more severe disease in humans and livestock than do Old World alphaviruses, these structural differences may be related to the pathobiology of the virus. In addition, conformational differences in the nucleocapsid may influence virus entry, disassembly, assembly, and/or budding.

Future cryo-EM studies of hazardous viral pathogens

The ability to visualize virus structures has been extremely important in understanding virus replication, assembly and pathobiology. Cryo-EM has provided numerous intermediate resolution structures of icosahedral viruses that are generally considered low health risk pathogens (see review: [21]). However, most microbes classified by the Centers for Disease Control and Prevention as category A, B and C pathogens have not been pursued structurally due in part to the difficulty in examining these pathogens. In elucidating the structures of pathogenic viruses by cryo-EM, we will need to confront several challenges.

The first challenge is to produce highly purified and concentrated virus samples. Generally speaking, 10^{10}–10^{11} particles per ml yield sufficient particle density per micrograph to enable an intermediate resolution structure to be efficiently determined by cryo-EM. Less concentrated virus samples require a significantly longer time to record the 5,000–10,000 particle images needed for an icosahedral particle reconstruction below 10 Å resolution.

The second challenge is the lack of icosahedral symmetry in many category A–C viruses, thus requiring reconstruction algorithms that are not dependent on the symmetry of the object for structural determination. Without particle symmetry to improve the signal-to-noise ratio of the reconstruction, $\sim$60 times more particle images are required to produce equivalent resolutions from asymmetric virus particles. Robust algorithms to perform such reconstructions have recently been developed and used successfully to determine the structure of non-viral particles to subnanometer resolution [9].

The third challenge is possible structural heterogeneity of the particles. It is conceivable that viral particles are dynamic and exist as an ensemble of structural conformations. This structural heterogeneity will limit the resolution of any three-dimensional reconstruction. However, it is plausible that one could develop new software to computationally "purify" particles that occur as discrete states within conformational ensembles. Because of the relatively large size of the virus particles and the possible continuum sampling of structural conformers, such an approach will present new challenges in the computational approach both at the level of algorithm development and implementation.

The fourth challenge in working with highly infectious particles is operator safety, since the particles maybe biologically active when placed inside the electron cryomicroscope. In a typical sample preparation, each cryo-EM grid would contain over a million of virus particles which are frozen, hydrated and can become aerosolized simply by allowing the grid to thaw inside a laboratory environment. Therefore, special specimen handling procedures for cryo-specimen preparation and transfer into the microscope column need to be developed to fulfill stringent safety guidelines. Furthermore, the maintenance of the instrument by microscope engineers also requires new protocols to ensure safety during instrument maintenance. Active research in robotic methods to handle specimens and microscope operation may yield a practical solution to safety issues within biosafety laboratories.

It is anticipated that in the near future active research programs investigating structures of highly pathogenic organisms will yield technical solutions to the above challenges. For instance, safety issues can be addressed by placing electron cryomicroscopes in BSL3 and 4 facilities so that biologically active viral particles can be examined at the highest possible resolution while remaining preserved in a completely contained environment. By determining the three-dimensional structures of important viral pathogens, we can build a structural encyclopedia of important pathogens. Such information can have a number of useful applications, including provisional viral identification during an outbreak, structure-based design of novel therapeutics and vaccines, and understanding molecular mechanisms of virus assembly and disassembly. The utility of such a research program will undoubtedly contribute to the overall effort in biodefense research in this country.

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Species barriers in prion diseases – brief review

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Summary. Transmissible spongiform encephalopathies (TSEs or prion diseases) are neurological disorders associated with the aggregation of a pathologic isoform of a host-encoded protein, termed prion protein (PrP). The pathologic isoform of PrP, termed PrP^{Sc}, is a major constituent of the infectious agent. TSE diseases are characterized by neurodegenerative failure and inevitable morbidity. Bovine spongiform encephalopathy (BSE) has been transmitted from cattle to humans to cause a new variant of Creutzfeldt-Jakob syndrome. The potential for chronic wasting disease to similarly cross the species barrier from cervids to humans is considered unlikely but possible. Thus, understanding how TSE agents overcome resistance to transmission between species is crucial if we are to prevent future epidemics. The species barrier usually can be abrogated to varying degrees in laboratory animals. Studies done with transgenic animals, tissue culture, and cell-free assays established PrP as being necessary for TSE pathogenesis and illustrated that certain amino acid residues are more influential than others for conferring resistance to TSE agent transmission. The essence of what constitutes a TSE agent's species compatibility is thought to be orchestrated by a complex interplay of contributions from its primary amino acid sequence, its glycoform patterns, and its three-dimensional structure.

TSE diseases

Prion diseases, collectively known as transmissible spongiform encephalopathies (TSEs), are a group of rare neurodegenerative syndromes in humans and other mammals for which there is currently no cure. The current state of knowledge has been well reviewed [1, 12, 14, 54]. The term spongiform is derived from the observation that the brain of an afflicted host is often scarred by intense neuronal vacuolation. Numerous TSE diseases have been characterized, including Creutzfeldt-Jakob disease (CJD) [59], Gerstmann-Sträussler-Scheinker (GSS) syndrome and kuru in humans [22], scrapie in sheep [15, 32], chronic wasting disease (CWD) [61] in deer and elk, and bovine spongiform encephalopathy

(BSE) [67]. Human TSE diseases exist as sporadic, inherited and acquired forms [75]. Sporadic TSE diseases, such as CJD, are the most common form of human TSEs and occur spontaneously in about one per million people annually, typically without clear evidence of exposure to an infectious agent. Inherited TSE diseases, which have been linked to more than 20 identified mutations in the gene that encodes for the prion protein, are extremely rare and have been documented in only a few families worldwide [21]. Acquired TSE diseases are divided into those infections obtained through the diet, such as kuru or vCJD, and those iatrogenically acquired from such activities as neurosurgery, growth hormone therapy and perhaps blood transfusions [40]. Infectivity for each of the major human TSE categories has been established by the successful transmission of disease to mice and/or non-human primates either by ingestion or injection.

A mammalian cell-surface glycoprotein termed prion protein (PrP^C) is involved in TSE pathogenesis. Human PrP^C is expressed at high levels in neurons and is translated as a 254 amino acid protein [46]. A 22 amino acid signal peptide is cleaved and a glycosylphosphatidylinositol (GPI) anchor is added post-translation. Functional PrP^C is normally anchored to the cell membrane. There are two asparagine-linked glycosylation sites found at residues 181 and 197. The ubiquitous nature of PrP and its prominent location on the cell-surface suggest that PrP provides a critical function for the organism, perhaps in copper metabolism or molecular signaling, but this function has yet to be elucidated [39]. One of the most significant milestones on the road to understanding the TSE phenomenon will be to decipher the physiological role of PrP.

TSE diseases are characterized by the accumulation of proteinaceous material primarily in the nervous system but sometimes the lymphoid system of the infected host. A predominant component of these aggregates and, indeed, of the infectious material is an abnormal isomer of prion protein (PrP^{Sc}) that is derived from the normal host-encoded cellular PrP^C. Current dogma dictates that aggregates of PrP^{Sc} act as a conformational template, first binding to a PrP^C molecule and then prompting its conversion into more PrP^{Sc} [33]. During this process, PrP^C changes from a soluble protein rich in α-helical structure into the abnormal PrP^{Sc} conformer, now less soluble and remarkably high in β-sheet structure [11, 47]. PrP^{Sc} differentiates itself from PrP^C by its relative insolubility in standard buffers, its notorious recalcitrance to proteolytic digestion [28], and its polymeric nature [10]. Formation of PrP^{Sc} is thought to be essentially irreversible, especially once it accumulates to high levels in the lymphoreticular and central nervous systems of the infected host. It is not known whether molecular species other than PrP are needed for PrP^{Sc} formation to occur.

Treatments designed to kill bacteria and viruses, such as germicidal light, alcohol, formaldehyde, and high temperature usually have a perplexingly negligible ability to inactivate TSE agents. Indeed, it has been shown that the TSE agent from the crude brain tissue of scrapie-infected hamsters can withstand temperatures of up to 600 °C and still preserve the ability to infect weanling hamsters [5]. Furthermore, no virus or bacterial organism has ever been shown to be a necessary

component of infectivity, thus supporting the hypothesis, postulated more than 35 years ago, that the pathogenic entity in these diseases is a self-replicating protein [26]. PrP^{Sc} has since been proposed as the infectious protein underlying TSE pathogenesis [55]. However, it has never been conclusively demonstrated that infectivity can be generated *de novo* without using material from an infected animal to trigger formation of new PrP^{Sc}. Even highly purified preparations of infectious material contain detectable amounts of molecular species other than PrP^{Sc}, including nucleic acid [66]. If some form of PrP itself is the sole source of infectivity, then we should be able to confirm this by initiating infection from a source that certifiably contains nothing but PrP. The fact that this has not been accomplished is a significant obstacle to the widespread acceptance of the protein-only theory of TSE infectivity. A key factor in our inability to test this theory is that we currently have no *ex vivo* method of generating significant amounts of properly glycosylated, appropriately misfolded and protease-resistant PrP from a non-infected or non-mammalian source.

Transmission of TSE disease

Intraspecies transmission

Intraspecies transmission of TSE infectivity is usually much more efficient than interspecies TSE transmission (Fig. 1). This is the case both in natural populations of animals with susceptibility to prion diseases and in experimental animals. Transmission efficiencies can nonetheless be quite variable depending on the TSE agent, the route of infection and the animal species. In the case of BSE, which is acquired orally with an incubation time of about 5 years, vertical or horizontal transmission is rare if it occurs at all.

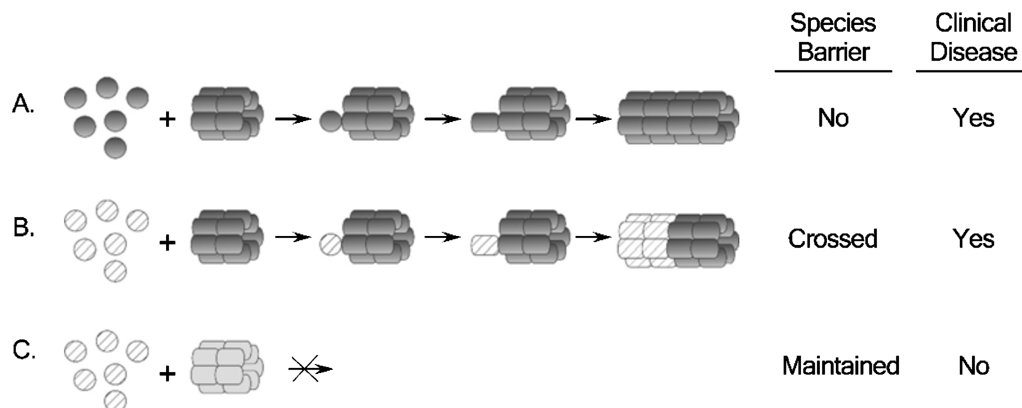

Fig. 1. Interactions between PrP^{C} and PrP^{Sc} influence TSE species barriers. Circles represent PrP^{C} and rectangles represent fibrillar PrP^{Sc}. (**A**) No species barrier. PrP^{C} and PrP^{Sc} are homologous. (**B**) Species barriers crossed. Host PrP^{C} and PrP^{Sc} inoculum are heterologous but match at key locations. Conversion occurs, and leads to PrP^{Sc} accumulation and disease. (**C**) Species barrier maintained. Host PrP^{C} and PrP^{Sc} inoculum are heterologous to the extent that successful conversion to nascent PrP^{Sc} cannot take place

Chronic wasting disease and scrapie are particularly intriguing TSE diseases because they are both clearly infectious without human intervention and neither disease has been traced to an anthropogenic origin. CWD is characterized by severe weight loss accompanied by extreme thirst. CWD spreads rapidly in captive deer populations, displaying infection rates of 80% or higher once CWD enters the herd [65]. The route of dissemination in the affected cervid populations is poorly understood, but it is thought that exposure to increased amounts of urine and saliva of affected animals may be involved in disease transmission. Similarly, scrapie is naturally transmitted between sheep, with maximum infection efficiencies anywhere from 25–40% [30].

BSE, on the other hand, is inefficiently transmitted if at all between cattle [80]. The likelihood that BSE came from an etiological source not evolved from the cow's natural setting may contribute to the relatively lower efficiency of BSE transmission between cattle. The underlying mechanism responsible for the intraspecies transmission characteristics observed in the animal's natural habitat are precisely the differences that must be understood in order to control the spread of TSE disease within animal populations.

The species barrier

Transmission of TSE agents between different mammalian species is limited by a "species-barrier" [48]. The phenomenon refers to the difficulty in transmitting a TSE agent from one species to another, and can be characterized by extended incubation times which notably decrease as the TSE agent is passaged through individuals of the recipient species. This impediment to interspecies transmission is sometimes so formidable that even intracerebral inoculation fails to produce clinical symptoms (Fig. 1). The barriers are even more pronounced when transmission is attempted by a less efficacious method, such as peripheral injection or oral inoculation. It is difficult to assess whether there are instances in which the species barrier has been circumvented in wild animals without human intervention. For example, CWD may or may not have arisen from exposure to sheep scrapie.

Concern about the transmission of animal spongiform encephalopathies to humans is now dramatically elevated because of the BSE epidemic in cattle. BSE stands as the classic and perhaps the only example of TSE agent transmission to humans from another species. The source of BSE infection in the United Kingdom is thought to be sheep scrapie contamination resulting from the now illegal practice of feeding cattle with rendered sources of mammalian ruminant protein, such as meat and bone meal, derived from the offal of cattle, sheep, and pigs as a high-protein nutritional additive. Overall, more than a million cattle are thought to have been infected. When BSE first ravaged Great Britain's cattle population in the late 1980s and early 1990s [53] there was some concern that humans might be vulnerable to BSE [69]. This unease was mitigated by the recognition that humans are not generally susceptible to sheep scrapie, which had been documented in England for more than two centuries. However, a few years after the BSE epidemic

peaked in 1992, a new form of CJD, termed variant CJD (vCJD), surfaced [76]. Consistently strong epidemiological, PrP^{Sc} typing and experimental transmission data support the hypothesis that vCJD emanated from BSE in cattle, although it is not well understood how BSE from cattle could cross the species barrier to humans by ingestion [6, 13].

It is not known how many people unwittingly consumed parts from BSE-contaminated animals; however, Britain's population of about 60 million people and all visitors during the peak of the BSE epidemic are considered to be at risk. Through careful surveillance, approximately 170 cases of vCJD have been documented since 1996. The epidemic appears to be on the decline. A relatively small fraction of apparent BSE exposures actually progressed to vCJD, thereby quantifying the dimensions of the species barrier between humans and cattle and illustrating the poor transmissibility of BSE to humans by ingestion. It is not known if a second wave of vCJD cases in hosts with longer incubation times will materialize.

Amidst the scientific and political landscape of the BSE problem, chronic wasting disease in deer and elk populations continues to spread across the central regions of North America [61, 77, 78]. The potential for transmissibility of CWD to livestock is a major concern because wild game and cattle often share the same pastures. No cases of CWD transmission from deer or elk to humans have been reported, but that is not to say that CWD cannot one day evolve into a distinct TSE agent that can infect humans.

There are at least two other situations whereby interspecies transmission of TSE diseases are thought to have occurred as a result of human intervention. Feline spongiform encephalopathy (FSE) has been documented in Norway, Ireland, Lichtenstein, and Great Britain [65]. Transmissible mink encephalopathy (TME) [42] has sporadically appeared in several countries where mink are farmed, including Canada, the USA, Finland and Russia. The epidemiological origins of TME and FSE have not been conclusively established; although TSE-contaminated nutritional supplements are strongly suspected [65].

Early experimental TSE transmission between species

In 1966, the cannibalism-related TSE disease kuru was transmitted to chimpanzees by intracerebral inoculation, with incubation times consistently less than two years [20]. Subsequent passages of brain homogenates from the sick monkeys to healthy monkeys of the same species resulted in progressively shorter incubation times [19]. In 1968, the first experimental transmission of CJD to chimpanzees was accomplished [23] and by 1973, the same researchers had expanded their work to include many species of monkeys [24]. Early researchers never doubted that the infectious agent was a virus, but were perplexed that CJD and kuru could be easily transmitted to certain species of monkeys but not to others [24]. Rhesus monkeys and cynomolgous macaques, for example, appeared immune to infection. Similarly, sheep and goat isolates of scrapie readily infected some species of monkeys but not others [15] while sheep scrapie could readily be transmitted to

mink and subsequently passaged in goats and hamsters [34]. A common theme among the early animal experiments was the inherent difficulty in trying to predict which species would succumb to a particular TSE agent. Nonetheless, these early experiments on the dissemination of TSE agents between humans and laboratory animals provided researchers with an important foundation upon which to build a mechanistic understanding of the species barrier.

The PRNP gene sequence influences the species barrier

Dickinson and coworkers originally reported that the host-encoded *Sinc* gene was influential in host incubation time and susceptibility to scrapie [16]. It was later determined from genetic crosses between C57BL and VM mice that the *Sinc* gene was the basis for the observed differences in incubation times [9]. Some scrapie isolates have longer incubation times in VM mice than in C57BL mice, but these differences could be controlled by selective breeding. When purified PrP was successfully analyzed by N-terminal sequencing, researchers were subsequently able to use DNA probes to establish that hamster cells contain a gene for PrP [46], now commonly termed PRNP. This same gene was isolated from mice and since then it has been established that all mammals examined to date contain a single chromosomal copy of the PRNP [41]. More recent experiments have confirmed that PRNP and *Sinc* are actually the same gene [44] and that amino acid sequence polymorphisms within PrP account for the difference in mouse scrapie incubation times. Different mammalian species also encode unique PrP sequences and thus the PRNP gene was rapidly subjected to closer scrutiny for its role in the determination of species barriers.

In the mid-1970's, an efficient Syrian hamster scrapie model was developed in which hamsters inoculated with scrapie became sick after only about 2–3 months [34, 43]. The hamster scrapie strain isolated, 263K, is considered nonpathogenic for mice thus making the transmission barrier between hamsters and mice a convenient experimental model [35]. With the advent of transgenic technology, the groundwork was laid for an intensive investigation of the species barrier between hamsters and mice. Initial experiments demonstrated that transgenic mice overexpressing Syrian hamster PrP succumb to illness within three months of inoculation with hamster scrapie whereas wild-type mice did not [62]. The post-inoculation incubation time was shown to be inversely proportional to the expression levels of hamster PrP^{C} in the brains of the transgenic mice, strongly suggesting that PrP^{C} was influencing disease kinetics [56]. Brain extracts from these "hamsterized" transgenic mice were also pathogenic for hamsters but not wild type mice on subsequent passage, suggesting that the specific characteristics of this TSE agent were retained between passages [56]. In a more recent but analogous set of experiments, it was established that incubation times for sheep scrapie in mice that overexpressed ovine PrP were also dramatically decreased relative to wild type mice [73]. Similarly, transgenic mice expressing human PrP^{C} were highly susceptible to human prions as expected [70]. The fact that the species barrier could be circumvented by changing nothing but the amino acid sequence

of PrP provided strong evidence that the PRNP gene and therefore PrP^{C} itself was a fundamental requirement for cross-species TSE transmission.

Because the primary amino acid sequence of PrP had been demonstrated as important for cross-species transmission, it was important to map the specific regions involved. A pioneering study was accomplished by Scott and coworkers with a construct in which a central portion of the mouse PrP open reading frame was replaced with the corresponding sequence from the Syrian hamster PRNP gene [63]. The chimeric mice were susceptible to infection from both hamster and mouse prions. A primary conclusion of this study was that this central region of PrP has the strongest influence on the TSE species barrier.

As further evidence of the crucial role of PrP in TSE diseases, it was eventually demonstrated that mice devoid of PrP ($Prnp^{0/0}$ mice) appear to live normal lives while demonstrating an apparently thorough resistance to TSE infectivity [7, 8] and an inability to propagate prion infectivity [60]. The question of how the $Prnp^{0/0}$ mice were able to function as normally as they did without PrP remains an enigma, although some $Prnp^{0/0}$ mice displayed sleep abnormalities [72] while others had increased levels of oxidative stress markers [79]. Nevertheless, the $Prnp^{0/0}$ mouse studies provided a critical validation of the research that had been done with hamsters and mice, suggesting that the PRNP gene, and therefore PrP, was an essential component of TSE pathogenesis.

Specific amino acids influence transmissibility of TSE disease

Once it was established that PrP was necessary for TSE pathogenesis and species barriers, further studies were directed towards finding more specific regions and particular amino acids in the PrP polypeptide that were the most influential in the conversion of PrP^{C} to PrP^{Sc}. Experiments with transgenic mice, tissue culture and cell-free systems all suggested that homology between PrP^{C} and PrP^{Sc} within the central region of PrP was important for the successful cross-species conversion of PrP^{C} to PrP^{Sc} and the transmission of TSE infectivity [38, 51, 63, 64, 71].

A direct correlation between the PrP amino acid sequence and TSE incubation time was demonstrated in transgenic mice expressing mouse PrP with amino acid substitutions Leu108Met and Val111Met. When challenged with mouse-adapted scrapie, these mice became sick after a significantly prolonged incubation time compared to mice expressing only mouse PrP [68]. Similarly, a single mutation in the N-terminus of mouse PrP (Pro101Leu) gene was found to significantly change the incubation times in transgenic mice inoculated with human, hamster and sheep strains of TSE [2].

When hamster PrP^{C} was expressed in scrapie-infected mouse neuroblastoma (Sc^{+}-MNB) cells, homology at residues 112–189 was required between PrP^{C} and PrP^{Sc} for the formation of nascent PrP^{Sc} [51]. A further finding in this study was that a mouse specific isoleucine (Ile138) facilitated the conversion of mouse PrP to its protease resistant form, while a hamster-specific amino acid (Met138) at that position prevented PrP^{Sc} formation [51]. The fact that a single amino acid mismatch was sufficient to prevent the PrP^{C} to PrP^{Sc} conversion in

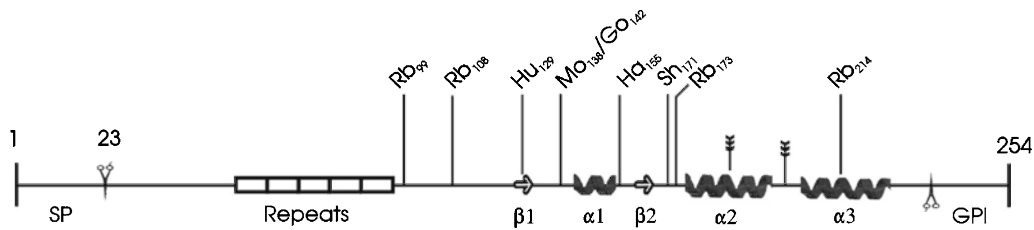

Fig. 2. Amino acid residues which are highly influential in maintaining and abrogating species barriers. The line represents full-length PrP^C; residues 1–254: *SP* signal peptide, *boxes* octapeptide repeats, *GPI* glycosylphosphatidylinositol anchor, added after cleavage of amino acid residues 231–254. The beta sheets are depicted by arrows and the alpha helices are shown on the main line. The central region of PrP is generally the most influential in terms of determining species barriers, however the specific amino acids responsible for this phenomenon vary between species. Residue numbers differ slightly between species: *Rb* rabbit, *Ha* hamster, *Mo* mouse, *Hu* human, *Sh* sheep, *Go* goat

the neuroblastoma cells suggested that there was exquisite amino acid sequence specificity needed for this process.

A single mismatch at position 155 (mouse Tyr154/hamster Asn155) between mice and hamsters in cell-free conversion experiments was sufficient to control the species-specific formation of protease-resistant hamster PrP [50]. Mouse PrP^C with the hamster specific Tyr154Asn mutation, grown in tissue culture cells and isolated by immunoprecipitation, was converted to its protease-resistant isoform much more efficiently than was the corresponding non-mutated mouse PrP. The conclusion was that residue 155 was highly instrumental in effecting the species barrier between hamsters and mice. In a similar cell-free experiment, a mismatch at PrP residue 171 in sheep significantly reduced the formation of protease-resistant sheep PrP [4], suggesting that polymorphism at this location could confer a prophylactic effect in sheep against scrapie. Taken together, these studies demonstrated that the barrier to interspecies TSE transmission can be enhanced by mismatches in key amino acid residues, but that the most influential amino acids are not always the same in every species (Fig. 2).

Heterologous PrP molecules interfere with the formation of PrP^{Sc}

Experiments with transgenic mice provided an abundance of evidence that many characteristics of TSE diseases, such as incubation times and neuropathology, could be modified by the PrP transgene [62]. In a study conducted with transgenic mice expressing either hamster or mouse PrP, it was shown that the PrP^{Sc} inoculum, whether from mouse or hamster, was a crucial factor in determining disease transmission between the two species [56]. In general, the greater the degree of homology between PrP^C and PrP^{Sc}, the more likely it was that cross-species TSE transmission would occur. A key observation in the transgenic mouse experiments was that mice expressing both hamster and mouse PrP were more resistant to mouse scrapie than were mice expressing only mouse PrP [56].

The possibility that expression of heterologous PrP^C, such as that from a different species, could interfere with formation of PrP^{Sc} prompted researchers to test this hypothesis in scrapie-infected murine neuroblastoma cells. These cells express murine PrP^C while at the same time accumulating and replicating mouse PrP^{Sc} and mouse scrapie infectivity [49, 58]. This model was efficaciously used to demonstrate that co-expression of both mouse and hamster PrP^C could significantly decrease the accumulation of PrP^{Sc} [49]. Heterologous hamster PrP^C interfered with the interactions between homologous mouse PrP^C and PrP^{Sc} in a dose-dependent manner, presumably by binding to PrP^{Sc} [29], as evidenced by the decreased production of proteinase K resistant PrP [49]. As the level of heterologous PrP^C expression was increased, the level of detectable PrP^{Sc} progressively decreased, eventually to an undiscernable amount. Cell-free conversion assays done *in vitro* [37] provided further evidence that heterologous PrP^C could not be converted to PrP^{Sc} with the same efficiency as that achieved with same-species PrP^C and PrP^{Sc} [38]. The overarching conclusion from these competition studies was that heterologous PrP^C molecules could help to prevent homologous PrP^C from misfolding into a proteinase K resistant form. This may be one of the decisive factors leading to the increased incubation times observed between species *in vivo* [56]. These experiments all provided further evidence that very specific intermolecular contact between the normal and misfolded PrP was needed to effect conversion [29].

Resistance to TSE infection?

Some species, such as guinea pigs and rabbits, have somehow evolved to be relatively free of TSE disease while other animals such as humans and sheep have not. This apparent resistance to TSE infection may be at least partially attributable to the primary amino acid sequence in the TSE-resistant animals. The rabbit, for example, appears to be TSE-resistant when subjected to intracerebral inoculation with TSE agent from different species [24]. Selected rabbit-specific mutations in mouse PrP, most notably Asn99Gly, Leu108Met and Asn173Ser, and Val214Ile were tested in Sc^+-MNB cells persistently infected with mouse-adapted scrapie agent and found to significantly interfere with the conversion of PrP^C to PrP^{Sc} [74]. These results, based upon conversion efficiencies in mouse neuroblastoma cells, were interpreted to mean that the previously documented resistance of rabbits to TSE infection was at least partially due to PrP^C-PrP^{Sc} mismatches at those amino acids.

Naturally occurring polymorphisms are often associated with enhanced susceptibility or resistance to TSE infection. For example, codon 129 in humans is associated with susceptibility to CJD [59] and an Ile142Met polymorphism in goat PrP is associated with resistance to both sheep scrapie and BSE infection [25]. It is well known that susceptibility to scrapie infection in sheep is determined primarily by residue 171 of ovine PrP [45]. Sheep that are homozygous for glutamine at codon 171 (171Glu,Glu) are more susceptible to scrapie than are their heterozygous (171Glu,Arg) or homozygous (171Arg,Arg) counterparts [45].

The observation that enhanced resistance correlates with specific point mutations has led some researchers to speculate that resistance alleles can be introduced into livestock by selective breeding and has prompted the implementation of breeding programs to eradicate scrapie in countries of the European Union [17, 31]. In fact, this strategy has been pursued in the case of sheep challenged with BSE. However, it appears that even sheep with the resistant phenotype succumb to BSE, albeit at a much slower rate [31]. Recent evidence shows that even very strong TSE barriers can eventually be crossed and it is debatable whether or not impermeable TSE barriers even exist [27, 57].

Factors other than the PrP sequence influence transmissibility of TSE diseases

Glycosylation

PrP is a cell surface glycoprotein, but the solved structures do not reveal any information about the effects of glycosylation or its interaction with the cell membrane on the function and conformation of PrP. Specific glycosylation almost certainly influences folding and thus TSE agent differentiation. Normal PrP is post-translationally modified by the addition of N-linked glycans at two asparagine residues near the C-terminus. However, the notion that a PrP molecule can be modified by only one or two well-defined sugar residues is vastly oversimplified. Indeed, the carbohydrate motifs themselves display considerable heterogeneity, with potentially hundreds of diverse carbohydrate structures [18]. Therefore, even PrP molecules with identical primary amino acid sequences can differ substantially at the molecular level. Indeed, it has been shown that glycosylation can have profound effects upon the amount of cross-species PrP^{Sc} (hamster and mouse) formed in an *in vitro* assay [52]. Successful models for the prediction of species barriers must someday address this issue of glycoform contribution to structure and pathogenicity. Thus, not only the primary amino acid sequence, but also glycosylation patterns are variables which undoubtedly influence key aspects of binding and the overall fold of both PrP^{C} and PrP^{Sc}.

Three-dimensional structure

A central tenet of structural biology has been that every protein molecule adapts a particular three-dimensional structure based upon its primary amino acid sequence. Prions represent an enigma in this respect because of their apparent ability to adopt numerous stable conformations based upon a singular primary amino acid sequence. Ultimately, the issue of TSE agent differentiation and transmissibility must be thought of in terms of its conformational variations from the overall PrP structure. One would expect that the highly conserved C-terminal domain of PrP (residues 121–231), for which there is about 90% sequence identity among mammalian species, should have very similar folds in healthy mammals. Indeed, this prediction is generally supported by the available

structures of recombinant PrP [36, 81–83]. When several important PrP surface residues from different species were analyzed in the context of existing biochemical data, the conclusion was that exchange of amino acids at these sites does not perturb the overall structure to any appreciable degree [3]. However, interchange of those same amino acids, by virtue of their promiscuous surface location, would display quite different hydrogen bonding and electrostatic properties, which in turn would significantly affect intermolecular communication at those sites. By this logic, it is conceivable that specific amino acids on the PrP molecule could be exchanged without loss of function, while at the same time affecting the way that PrP^{C} interacts with other molecules; for instance, PrP^{Sc} from another species.

Despite the apparent consistency of non-glycosylated PrP^{C} structures between species, the basis for TSE agent specificity and the species barrier almost certainly relates to subtle variations in the three-dimensional structure of PrP^{Sc} [74]. The currently available structures of PrP do not include PrP^{Sc}, which has architectural features dramatically different from PrP^{C}. The question of whether variations in PrP^{Sc} three-dimensional structure lead to the creation of distinct TSE agents, or if such conformers are generated as a molecular repercussion of the underlying disease process, has yet to be resolved.

Conclusion

The transmission barrier for TSE infectivity following passage from one species to another can be manifested either as an extremely long incubation time prior to onset of clinical signs or as a complete lack of clinical disease. It remains difficult to predict the magnitude of this barrier. This is especially true if transmission occurs across multiple species as may have happened with BSE when it apparently adapted from sheep to cattle to humans. It is conceivable that deer and elk harboring CWD could transmit to range cattle and expose the human population to a new strain of TSE. Reliable models for predicting the resistance of humans to animal prion diseases are needed. A dire need in the field of prions is the availability of a cost-effective, quick and minimally invasive diagnostic tool to monitor the early occurrence of TSE in human and animal populations. The current method of testing livestock for TSE infection requires killing the animal before the actual test, making the testing expensive, laborious and inherently inefficient. The application of techniques from the rapidly developing field of proteomics to the analysis of TSE agent differentiation would be a welcome addition to the study of these diseases at the molecular level. Although considerable progress in prion research has been made, the paucity of diagnostic, preventative, and treatment options highlights the need for a more comprehensive mechanistic understanding of TSE infectivity.

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Academic science and the business of vaccines

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Many elements of the global medical research and development enterprise are involved in the discovery, development, manufacture, distribution and regulation of vaccines. These include academic scientists, government funding and regulatory agencies, commercial vaccine manufacturers in the western world and essentially generic vaccine manufacturers in the developing world. The central tenets of the system as it exists today are 1) that public health need will drive fundamental research supported by government at academic institutions, and 2) that the resulting discoveries will be translated into useful products, because public health need can be converted into an economic return for the vaccine industry and its shareholders. In those instances where public health need exists in parallel with potential economic return, the system works surprisingly well.

For diseases primarily affecting resource poor areas of the world, such as AIDS, malaria, tuberculosis and dengue, the causative agents have been known for decades, yet no affordable, effective vaccines exist. One reason for this stunning lack of success is that substantial technical challenges are presented by these diseases. However, it is my opinion that the major cause for this failure is the absence of a parallel between public health need and potential economic return for diseases of developing countries, where the need is high but the projected economic return is invariably low.

All the participants in global vaccine discovery, development and manufacture are limited in ways that impede their ability to effectively address these vaccine needs. Commercial vaccine companies have a fiduciary responsibility to their shareholders to provide maximal returns on investment. Many of the largest vaccine companies are actually relatively small divisions of much larger conglomerates. These vaccine divisions compete for resources within the larger entity on the basis of internal rates of return calculated for each division. Given that vaccine manufacture is inherently less lucrative than some other endeavors in the medical arena, such as cancer drugs, the vaccine divisions must focus almost exclusively on products that will give a high monetary return. Thus, even at the earliest stage of vaccine development, choice of vaccine target is dictated by market size in dollars rather than by public health need. This selection criterion

almost always excludes vaccines for diseases primarily affecting countries with poor economies.

Universities are repositories of knowledge and new ideas, and are populated by persons generally inclined to contribute to the public good. Exciting discoveries in fundamental pathogenesis, immunology and genetics flow daily from our universities and government laboratories, and have led to several very promising new vaccine technologies. However, universities are not organized, chartered or otherwise suited to translate these technology concepts into actual products. The scope of their efforts rarely extends beyond the laboratory door.

To move their basic discoveries to application as products, most universities have now constituted technology transfer offices to patent their new vaccine technologies and prototype vaccines. However, these offices generally are chronically underfunded and lack the resources to sustain the patenting process without a commercial partner. This most often necessitates that a university technology be licensed at a very early stage of development and that the license be exclusive and worldwide. Because of these constraints, research universities inadvertently contribute to the market bias of the industry. When an academic institution licenses its discoveries to a commercial company for further development, it accepts the company's market-based decision-making criteria by default. Few if any options to this licensing process are open to universities.

There is an active and growing group of vaccine manufacturers in the developing world, and they produce most of the world's polio, measles and hepatitis B vaccines, for example. However, these low cost manufacturers operate like generic drug companies, depending for their product pipeline on commercial vaccine products coming off patent. Thus, there is significant delay in their ability to produce new vaccines. Because the pipeline was selected originally by the commercial manufacturers, vaccines for diseases predominantly of the developing world are not present in the product pipeline, even through this delayed process. Resource constraints limit these companies in terms of generating their own suite of in-house products.

Therefore, the useful fruits of academic science, overwhelmingly funded from government and philanthropic sources, are most often provided only to wealthy countries with little or no direct benefit to those nations most in need. The question then, for those of us in the vaccine research community is, "How do we insure that the benefits of our research are available to all those who might have need of them?"

We propose a new type of not-for-profit research and development entity, Global Vaccines, Inc. (GVI). GVI will be highly focused on vaccine technologies and their application to vaccines against diseases of the developing world. GVI will 1) Apply state-of-the-art vaccine technologies and innovative business strategies to the design and development of affordable vaccines for resource poor nations, 2) Provide an alternative strategy for university based technology licensing that will protect promising new vaccine technologies for use in poorer countries, 3) Develop these technologies in the context of prototype vaccines addressing global public health needs, and 4) Link academic vaccine research centers to independent

vaccine manufacturing capabilities already established in the developing world, facilitating the application of promising vaccine technologies as prototype vaccines.

Global Vaccines will utilize the business expertise, discipline and energy of an entrepreneurial business to circumvent many of the current limitations of the western vaccine industry, vaccine manufacturers in the developing world, and academia. The new company will have the flexibility to devise innovative solutions to vaccine problems of the developing world, not only in the laboratory, but also in licensing vaccine technologies and financing development and production of these vaccines. The primary objective of GVI will not be to maximize profit but rather to maximize the likelihood that a given vaccine concept will reach successful application in the field.

Global Vaccines will develop platform vaccine technologies that can be applied to a broad spectrum of disease targets and will identify such potentially viable new vaccine technologies at the earliest possible stage, in some instances at the level of basic discovery. As with a commercial company, GVI will seek exclusive, worldwide rights in return for payment of an initial licensing fee and assumption of patent expenses. In contrast to most commercial concerns however, GVI and the university will remain partners in the development of the technology through proof-of-concept in appropriate animal models and perhaps through phase I trials in humans. Such partnerships will be led by GVI, will involve the original inventors and will be supported by jointly obtained research grant funds. By advancing a technology to proof-of-concept, the value of the technology will be far greater than at the time of the original license, thus enabling a sublicense to a commercial company with technology rights geographically limited to commercial markets. GVI will utilize the gains from this sublicense to subsidize continued development of needed vaccines, partnering with low cost manufacturers in the developing world. Note that a commercial sublicense and establishment of a partnership with

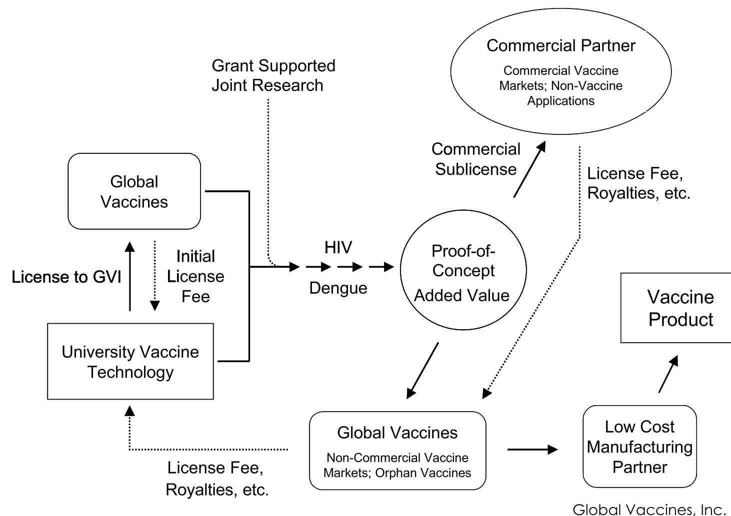

Fig. 1. GVI technology licensing and partnering strategy

a commercial vaccine manufacturer could occur at any point in the technology development timeline if circumstances were favorable.

As the licensing university will share in the proceeds of the commercial sublicense, the GVI strategy can potentially provide a much larger and more timely return for the university than could have been realized in a typical early license agreement with a for-profit concern. Therefore, GVI can compete very effectively with commercial companies in obtaining licenses for exceptional vaccine technologies.

The GVI strategy has the potential to benefit everyone. Vaccines will be provided to the populations most in need. Inventors and their universities will receive a fair return for their inventions. Developing country vaccine manufacturers will have a pipeline of high technology products for the populations that they serve. And, commercial vaccine companies will have the opportunity to license more mature and therefore less risky vaccine technologies for their use in profitable markets.

Global Vaccines has taken the first steps to position itself to implement this strategy. The company is constituted as a North Carolina corporation, and it has obtained tax exempt status from North Carolina and the Federal Government. Negotiations have begun for licensing the company's first vaccine technologies, and a major fund raising effort is being planned.

It is our hope that Global Vaccines will complement the many other ongoing efforts to alleviate health disparities between rich and poor populations, and that this new entity will ultimately contribute to a solution for these currently intractable diseases.

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Emerging infectious diseases: the public's view of the problem and what should be expected from the public health community

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The public's view of major threats to health, as with other contemporary issues, is largely influenced by the media. As new health-related information is released from the clinical and research communities, it is translated for and disseminated to the public through a variety of mechanisms. In the past, healthcare providers served as the primary source of health-related information for patients. Today, however, an unprecedented interest in health issues has led to intense media coverage of medical developments. Moreover, the internet has given interested individuals rapid access to virtually unlimited sources of information. Because of this symbiotic relationship between public interest and media attention, the actual impact or severity of a public health problem can be disproportionate to the amount of media coverage it receives, creating a climate of unnecessary fear and obscuring important health messages [1].

In 1999, the Centers for Disease Control and Prevention (CDC) released a series of reports describing ten great public health achievements in the United States during the 20th century [2]. The topics were chosen based on their impact on reducing death, illness, and disability in the United States, and include advances such as vaccinations, improved maternal and child health, safer and healthier food, fluoridation of drinking water, and safer workplaces. Also among this list is control of infectious diseases, resulting from improvements in sanitation, access to clean water, and the development and use of effective vaccines and antibiotics. So dramatic were these advancements that by the middle of the 20th century infectious diseases were no longer viewed as major public health threats in the United States and in many other developed countries. This false sense of security was short lived, however, as newly recognized and reemerging diseases continued to appear, many of which produced devastating consequences – most notably HIV/AIDS.

Advances against infectious diseases have not been universal. Worldwide, infectious diseases continue to be a leading cause of death, profoundly impacting the developing world. The World Health Organization (WHO) estimates that nearly 15 million (26%) of the approximately 57 million deaths that occurred

throughout the world in 2003 were caused by microbial agents [3] (Table 1). Leading the list are lower respiratory infections, responsible for 3.9 million deaths per year, followed by HIV/AIDS (2.8 million), diarrhea (1.8 million), tuberculosis (1.6 million), and malaria (1.2 million) [3]. The true burden of death from infectious diseases, however, is much higher since underreporting remains a major factor, particularly in the developing world. Moreover, many deaths associated with infections are not categorized as infection related (e.g., deaths from cancers caused by infectious agents). Despite the continued dramatic impact of these global killers, they receive very limited media attention – having become commonplace compared to the new and exotic.

One of the reasons for this disparity has been the actual increase in the number of emerging and reemerging infections that have surfaced during the last 10 years (Box 1). Examples include newly recognized diseases such as hantavirus pulmonary syndrome, new variant Creutzfeldt-Jakob disease, and Nipah and Hendra viral diseases, the introduction and spread of West Nile virus infection in North America, and intermittent outbreaks of Ebola hemorrhagic fever in parts of Africa. Other major concerns include the increasing problems created by antimicrobial resistance and the continued threat of bioterrorism. In 2003 alone, a newly recognized coronavirus spread across five continents sickening more than 8,000 people and causing 774 deaths from a new disease designated severe acute respiratory syndrome (SARS) [4], the exotic animal trade resulted in the first cases of human monkeypox in the Western hemisphere [5], and highly pathogenic strains of avian influenza virus killed humans and devastated the poultry industry in parts of Asia [6] – further heightening fears of pandemic influenza.

This continual onslaught of newly identified and reemerging infectious diseases, along with increased concerns on the part of policymakers, the media, and an interested public, has created a new public health perspective and a heightened sense of vulnerability regarding infectious diseases. Experiences with both naturally occurring and intentionally caused diseases have clearly demonstrated that infectious diseases can have severe consequences beyond public health, impacting national security and the global economy. Local outbreaks are no longer

Table 1. Leading infectious causes of death, 2003

Cause	Deaths
Lower respiratory infections	3.9 million
HIV/AIDS	2.8 million
Diarrhea	1.8 million
Tuberculosis	1.6 million
Malaria	1.2 million

Source: World Health Organization. The World Health Report 2004: Changing history. Geneva: World Health Organization, 2004

Box 1. Selected infectious disease challenges 1993–2004

Hantavirus pulmonary syndrome
Plague
Ebola fever
New variant Creutzfeldt-Jakob disease
H5N1 inuenza
Nipah virus encephalitis
West Nile virus infection
Rift Valley fever
Anthrax
Vancomycin-resistant *Staphylococcus aureus*
SARS
Monkeypox
Community-associated methicillin-resistant *S. aureus*

considered limited threats but rather sentinel events capable of having much wider and potentially catastrophic implications. As a result, rapid and collaborative responses to infectious disease outbreaks have become both essential and expected.

In 2003, the Institute of Medicine published a report highlighting the increasing risks to public health posed by emerging microbial threats [7]. The report, *Microbial Threats to Health: Emergence, Detection, and Response*, serves as an update to the Institute's 1992 landmark report on emerging infections [8], which issued a strong caution against complacency toward infectious diseases and called for a rebuilding of the nation's public health system. The new report categorizes the spectrum of microbial threats into five areas: the global burden of AIDS, tuberculosis, and malaria; antimicrobial-resistant infections; vectorborne and zoonotic diseases; chronic diseases with infectious etiology; and microbes intentionally used for harm. The report also describes more than a dozen factors – human, biological, social, and environmental – that can work alone or in combination to produce a global microbial threat. Examples of these factors include human demographics, behavior, and susceptibility to infection; changes in technology, industry, travel, and commerce; changing ecosystems and microbial hosts; and social and political factors such as poverty and other inequities, lack of political will, and the consequences of war and terrorism.

As if a portent, the release of the IOM report in March 2003 coincided with the outbreak of SARS. The disease would prove to be an archetype of a global microbial threat, spreading rapidly as a result of international travel and requiring an international response to stop its spread. Although the earliest notification about the illness came on February 10, 2003, through a report posted on the Program for Monitoring Emerging Diseases, or "ProMed" [9], the disease had been occurring in southern China since November 2002 – spreading largely to hospital workers who had treated affected patients. The global outbreak began on February 21, 2003, when a Guangdong physician, traveling while ill, spent one night in a Hong Kong hotel. Although the exact modes of transmission are unknown, this individual would infect more than a dozen other hotel guests and visitors, many of whom served as index patients for major outbreaks in Hong Kong, Singapore, Vietnam, and Canada [10] (Fig. 1). In Singapore, more than 170 of the country's 238 SARS cases were linked to a single individual who became infected at the Hong Kong hotel [11].

Much has been learned from these recent outbreaks of emerging infectious diseases, especially SARS (Box 2). Despite its tragic health consequences and strong social, economic, and political impact, SARS was fortunately not the feared "Big One," appearing to spread primarily by droplets during close contact. The SARS outbreak uncovered both strengths and weaknesses in global disease detection and response efforts and can therefore serve as a strong warning as well as an opportunity to prepare for future threats [12]. SARS clearly showed the unpredictability of emerging infectious threats and the vulnerability of even the most developed nations. The virus did not respond to treatment, and no vaccine was available. The use of strict isolation and quarantine precautions – some involving tens of thousands of individuals – proved the best means of stopping the epidemic.

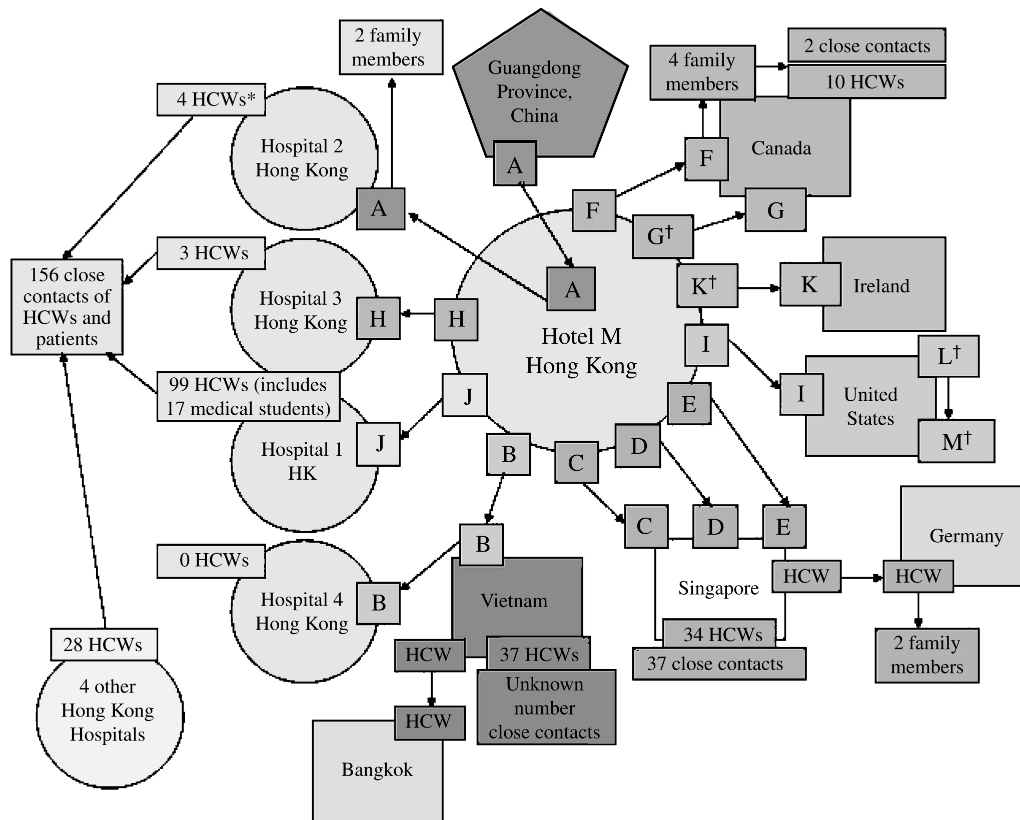

Fig. 1. Chain of transmission among guests at Hotel M – Hong Kong (2003): *Health-care workers; †Guests L and M (spouses) were not at Hotel M during the same time as index Guest A but were at the hotel during the same times as Guests G, H, and I, who were ill during this period. Data as of March 28, 2003

Box 2. Improving preparedness and response: *lessons learned from recent outbreaks*

– Strengthening existing and developing new national and international partnerships
– Training and educating a multidisciplinary workforce
– Ensuring "full use" of investments
– Encouraging transparency and political will
– Fostering a global commitment to address inequities
– Developing and implementing preparedness plans and research agendas
– Proactively communicating with health professionals, the media, and the public

While the first line of defense in controlling an outbreak remains strong national surveillance systems that can readily detect outbreaks, the SARS experience highlighted the importance of global disease detection efforts [13]. The same interconnected world that enables microbes to rapidly cross borders can also work to effectively stop their spread, providing an opportunity for establishing surveillance systems that can approach real time. For SARS, the internationally coordinated response led by WHO allowed clinical, research, and public health

experts around the world to exchange information on the new disease as quickly as it evolved. Part of this effort included the WHO Collaborative Multi-center Research Project on SARS Diagnosis, a network involving more than a dozen laboratories and 10 countries. In less than a month, three of these laboratories determined the cause of the illness – a previously unrecognized coronavirus. Also playing a major role in the response was WHO's Global Outbreak and Response Network (GOARN), a surveillance and response system of more than 120 organizations worldwide. Although GOARN responds to dozens of outbreaks in developing countries each year, the SARS outbreak represented its first response to an internationally spreading illness [13]. Among GOARN's most visible partners are the National Influenza Centers (http://www.who.int/csr/disease/influenza/centres2004/en/). Established in the 1950s, this expansive network of more than 100 institutions in over 80 countries is responsible for tracking influenza viruses to guide vaccine development and to recognize variants that may be capable of producing a pandemic.

Another message clearly indicated from recent emerging and reemerging infectious diseases is the need to strengthen existing and establish new linkages between the human and animal health communities. The majority of pathogens implicated in recent outbreaks, as well as most of those identified as potential bioterrorism agents, are vector-borne or zoonotic microbes, many of which have crossed the species barrier from animals to humans [4, 14] (Box 1). Continued urbanization and other environmental and human demographic changes suggest that this emergence of new zoonotic diseases will likely continue, requiring a corresponding convergence of highly trained human and animal health experts to effectively address them.

Ensuring that these experts have the capacity to respond to a broad range of infectious threats requires recruitment efforts and training programs across a variety of disciplines including clinical, laboratory, epidemiologic, and behavioral research. National and international collaborations among a skilled workforce are critical for improving global disease detection and ensuring an effective response. Such investments in human resources must also be met with improvements in research facilities and capacities. The benefits of such efforts can be substantial, extending beyond national borders and allowing for a "dual" or "full" use of resources. In the United States, investments made to strengthen national bioterrorism preparedness and response efforts over the past several years have improved overall preparedness for public health threats. An example is the Laboratory Response Network (LRN), a network of public and private laboratories established in 1999 by the Centers for Disease Control and Prevention (CDC) to respond quickly to acts of chemical and biological terrorism, emerging infectious diseases, and other emergencies. In 2003, the LRN provided valuable diagnostic services for SARS, monkeypox, and avian influenza, in addition to daily monitoring of potential bioterrorist agents.

The critical importance of transparency and political will in controlling infectious diseases was also evident during the SARS outbreak. China's months-long delay in reporting the outbreak not only prevented efforts to contain the epidemic locally but also proved most costly for its own region. In contrast was Vietnam, one

of the earliest countries affected by the outbreak and the first to contain it [15]. Dr. Carlo Urbani, an infectious disease physician working in Hanoi for WHO, recognized the unusual severity of the disease and quickly instituted infection control precautions, sadly too late to prevent his exposure to the infection that would cause his death. Dr. Urbani's prompt recognition along with Vietnam's commitment and global cooperation effectively limited the spread of SARS in Vietnam. China ultimately demonstrated one of the most extraordinary acts of political will in addressing the epidemic when more than 4,000 construction workers built a 1000-bed hospital in approximately one week. The importance of political will in addressing infectious diseases continues to be demonstrated most directly by its absence – an all too frequent obstacle to eradication efforts for vaccine-preventable diseases such as polio and measles.

Closely tied to political will is a commitment on the part of high income countries to help address inequities – the social, economic, and health disparities that contribute to the spread of infectious diseases [7, 16]. In 2000, at the United Nations Millennium Summit, representatives from nearly 200 U.N. member states resolved to help end human poverty and its ramifications. Termed the "Millennium Development Goals," this agreement requires countries to increase their efforts to address inadequate income; lack of food, clean water, and health care; substandard education; gender inequality; and environmental degradation. The goals also call for renewed commitment in addressing the disproportionate impact of infectious diseases on many of the world's poorest regions. A more recent undertaking is "The Grand Challenges in Global Health" initiative, funded by the Bill and Melinda Gates Foundation and administered by the Foundation for the National Institutes of Health. This initiative was established in 2003 to help develop solutions to critical problems that perpetuate the spread of disease in the developing world. Such international undertakings directed toward the diseases causing the greatest morbidity and mortality in the developing world should be priorities for wealthier countries. In addition to meeting enormous humanitarian needs, efforts to address these daunting global killers can help remove major obstacles to economic growth and development, thereby strengthening public health infrastructures and disease detection capacities worldwide.

Perhaps most evident during the SARS outbreak was the crucial need for rapid dissemination of accurate information – both for the medical and scientific experts confronting the epidemic and for a concerned public. During the SARS epidemic, the availability of electronic communications enabled networks of laboratory scientists, clinicians, and public health experts to share information and rapidly generate a scientific basis for public health action against a novel disease [17] – a major step toward lessening the health consequences of the outbreak. These extraordinary efforts and swift actions, however, did not prevent the severe social and economic ramifications that resulted from SARS. These consequences, largely generated by the fears and perceptions of a vulnerable public, highlight the critical need to communicate timely and accurate information in the face of scientific uncertainty. Proactive communications directed at health professionals can enhance the ability of those on the front lines to detect the

unusual – e.g., test results or patient symptoms that could signal the occurrence of a new health threat. Similarly, proactive and open communication between public health officials and policymakers is essential for sound public health action. Finally, proactive communications through public health websites and with the media can help ensure broad dissemination of timely and accurate risk information to members of the public that can enable them to make important decisions in protecting their health.

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