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BIOLOGICAL CHARACTERIZATION OF PLAQUE-SIZE VARIANTS OF YELLOW FEVER VIRUS IN MOSQUITOES AND MICE

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Summary. — We isolated plaque-size variants of a South American strain of yellow fever virus, and compared their ability to infect orally and be transmitted by vector *Aedes aegypti* mosquitoes with that of the uncloned, parental virus. We analyzed the same clonal isolates in mouse virulence experiments.

No significant differences could be demonstrated in the capacities of the variants to infect and be transmitted by mosquitoes or in mouse virulence tests. The 17D vaccine virus (derived from the African Asibi strain) was, however, markedly attenuated in mosquitoes; also, a variant virus (Asibi strain) derived by continuous passage in HeLa cells (and attenuated for monkeys and mice) was markedly attenuated in mosquitoes when compared with its parent virus. The results suggest that vector competence and mouse virulence are similar in plaque-size variants of the South American strain of yellow fever virus. However, if vigorous and appropriate selection pressures are applied (as established by the derivation of the 17D vaccine and HeLa passaged viruses), attenuated variants can be discovered; their role and prevalence within a virus population in nature is unknown.

Key words: yellow fever; Aedes aegypti; vector competence, virus variants

Introduction

The variability of animal viruses with RNA genomes is a well recognized phenomenon (Holland *et al.*, 1982). Genetic reassortment of viruses with segmented genomes and point-mutation are important mechanisms for ensuring the survival of virus strains in nature. The mutability of RNA viruses can, of course, generate genetic variants with altered biological characteristics that may influence the ability of the variant to replicate successfully in host cells. Clearly, the two most important factors to be considered in this context are mutation rates for viruses with nonsegmented genomes and selection pressures generated by the host's defense mechanisms. Arboviruses are

subject to selection pressures resulting from the necessity of replicating in cells with vastly differing phylogenetic histories, the vector's and the host's. What pressures (if any) are exerted by arthropod cells on genetic variants are largely unknown.

Recently, Miller and Mitchell (1986) suggested that the occasional variation detected between replicate experiments in vector competence studies with yellow fever virus occurs because the viruses used represent genetically diverse populations, and only a part of the population efficiently replicates in mosquito cells. Thus, the relative proportion of this subpopulaton in the infectious meal might explain the observed variation. We attempted to address this hypothesis by isolating and purifying plaque-size variants of yellow fever virus and characterizing them in terms of vector competence in mosquitoes and virulence in sub-adult mice. This information is essential for selecting a genetically defined population (plaque pick) that expresses a wild type phenotype in mosquitoes and mice for sequence analysis and selection experiments.

Materials and Methods

Viruses and cells. The strain of yellow fever virus (1899/81) from which the plaque-size variants were cloned, was a human isolate from Peru which had undergone 2 passages in *Aedes albopictus* (clone C6/36) cells and a single passage in suckling mice (Mendez *et al.*, 1984). Plaque size was defined as small (<1 mm diam.), medium (1–3 mm diam.), and large (>4 mm diam.) 9 days post inoculation in Vero cells. Plaques were picked with a Pasteur pipette and amplified in BHK-21 cells; this process was repeated 3 times, at which time suckling mouse brain virus stocks were prepared. Uncloned Asibi virus (passage history: 6 times in monkeys, twice in C6/36 cells, once in suckling mouse brain, and once in C6/36 cells) and its vaccine derivative, 17D (Connaught [Swiftwater, PA, USA] commercial preparation passed twice in suckling mouse brains) were used as controls in the vector competence experiments. A virus cloned from the Asibi strain (passage history: monkey-6, C6/36 cells-2 suckling mouse brain-1, C6/36 cells-1) and grown 6 times in HeLa cells and known to be attenuated for monkeys and mice was also included in the vector competence studies (T. P. Monath, D. Adkins and A. D. T. Barrett, unpub.; Converse *et al.*, 1971; Hardy 1963; Hearn *et al.*, 1966).

Virus was plaque-assayed in 60 mm plates seeded with Vero cells. Cell monolayers were inoculated with 10-fold dilutions of virus suspensions and incubated at 37 °C for 1 hr in a CO_2 (5 %) incubator. The monolayers were then overlaid with 8 ml of SeaKem (FMC, Rockland, ME) agarose (1 %) and Earle's BSS supplemented with 4 % heat inactivated (56 °C, 30 min) foetal bovine serum and 6.6 % yeast extract-lactalbumin hydrolysate. After 7 days incubation, a second overlay was added (2 ml) containing neutral red ($1 : 10^4$). Plaques were read 2 days later.

Mosquitoes and mosquito infection. Aedes aegypti mosquitoes (Rexville strain) were kindly provided by Dr. D. J. Gubler (Dengue Branch, Centers for Disease Control (CDC), San Juan, Puerto Rico). They had undergone 9 generations of colonization at CDC in Fort Collins, Colorado.

Mosquitoes were exposed to yellow fever virus by feeding on cotton pledgets soaked with a warm infectious meal (37.8 °C) consisting of equal volumes of washed human red blood cells (1 time in Alsever's solution, 3 times in phosphate buffered saline [PBS], pH 7.5) and virus. The virus was in the form of freshly harvested suckling mouse brains. One brain was triturated in 1 ml MEM with 2 % foetal bovine serum and centrifuged at 5000 rev/min for 15 min in a Sorval SS 34 rotor (Miller, 1987). Mosquitoes were exposed for 15 min, and fully engorged mosquitoes were sorted, placed in cages made from 1-gallon paper containers and incubated 14 days at 26.7 °C, 80 % RH.

To assay for virus transmission, we allowed surviving mosquitoes to bloodfeed on 2-day-old suckling mice. Mice were monitored for 14 days for signs of illness or death.

Mosquito infection with yellow fever virus was determined by examining head tissues (after 14 days' extrinsic incubation) for specific yellow fever viral antigen by immunofluorescence. The

fluorescein isothiocyanate conjugate was a CDC reagent (anti-Asibi, M15154, Lot 2, 1/19/81) and demonstrated a titer of 1 : 40. Mosquito "headsquashes" were made on standard microscope slides and fixed for 10 min in cold (−20 °C) acetone. Conjugated antibody was incubated with mosquito headsquashes for 30 min at 37 °C in a humidified chamber, rinsed once for 15 min in PBS and once in "buffered water" (PBS without NaCl) for 15 min, and air-dried. Evans blue was added to the conjugate as a counterstain. Slides were mounted in polyvinyl alcohol and examined with an Olympus BHC incident light fluorescent microscope a xenon light source, 490-nm exciter, and 530-nm barrier filters.

Virulence of yellow fever virus variants in mice. Ten-day-old, outbred, white Swiss mice were inoculated by the intracerebral (i.c.) and intraperitoneal (i.p.) routes with 10-fold dilutions of virus and observed daily for signs of illness and death as described by Barrett and Gould (Barrett and Gould, 1986). Ten-day-old mice were used in these experiments because a previous study by Fitzgeorge and Bradish (1980) showed this age allowed optimal discrimination of virulence among South American strains of yellow fever virus.

Data were statistically analyzed with 2 × 2 Chi-square tables.

Results

Mosquito infection and transmission rates of variant viruses

Figure 1 shows typical plaque morphology of the variant size classes, the uncloned parental strain, and the very large plaque of the vaccine virus (17D). Except for the vaccine virus, none of the size classes demonstrated 100 % uniform plaque morphology even after 3 plaque-purifications; nonetheless, the plaques reported for each size class easily represented the majority population (Fig. 1).

Aedes aegypti mosquitoes ingesting large amounts of the South American variant viruses became infected and were able to transmit the viruses via bloodfeeding on suckling mice at rates similar to those of the parental virus strain from which they were derived (Table 1). Virus transmission rates

Table 1. Infection and transmission rates in Aedes aegypti mosquitoes orally exposed to plaque-size variants of yellow fever virus

Virus Strain	Plaque Size*	Meal titer Log_{10} PFU/ml	No. disseminated Infection/No. exposed (%)	No. transmitting/ No. disseminated infection (%)
1899/81 Parent	Mixed	7.8	28/28 (100)	21/24 (88)
A2.1 (1)	Large	8.3	38/40 (95)	23/25 (92)
B3.1	Large	7.6	36/40 (90)	27/31 (87)
B1.1	Medium	8.0	37/40 (93)	22/27 (81)
A5.1 (1)	Small	8.0	28/28 (100)	11/16 (69)
A3.1	Small	8.2	39/40 (98)	25/30 (83)
B4.1	Small	8.0	39/40 (98)	31/31 (100)
Asibi	Small	7.8	31/33 (94)	12/17 (71)
17D	Large	7.2	1/32 (3)	0/1
Hela	Large	6.7	0/48	

* Small = < 1 mm; medium 1 − 3 mm; large > 4 mm; 9 days postinfection in Vero cells.

ranged from 69% (11/16) to 100% (31/31) for the plaque-size variants; however, none of these rates was statistically different from those of the parent virus with a mixed plaque morphology. Virus recovered from orally infected mosquitoes (14 days' extrinsic incubation period) retained the predominant plaque morphology of the infecting virus clone (data not shown).

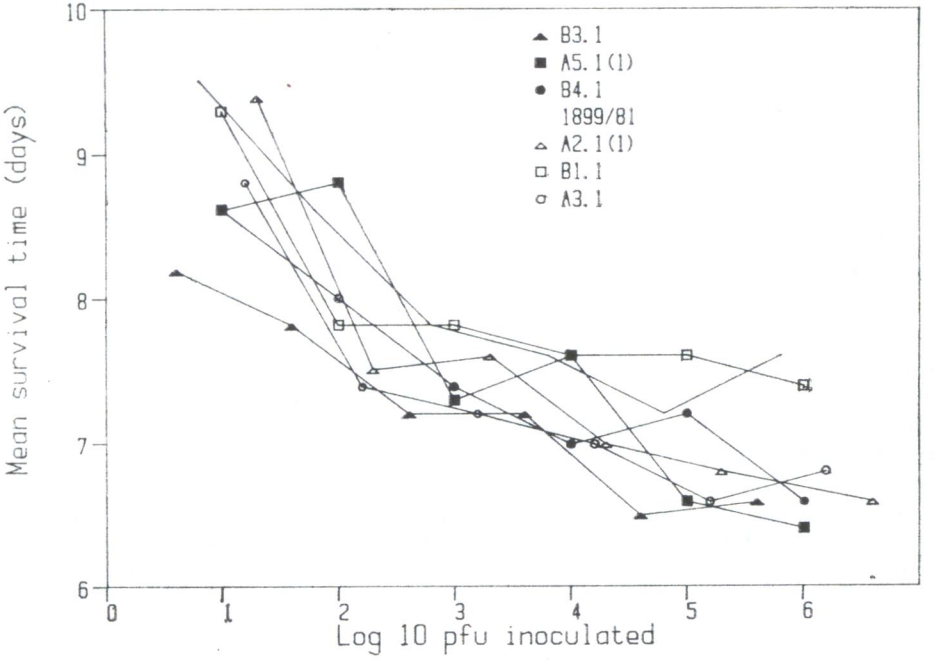

Fig. 2.
Survival times of 10-day-old mice inoculated intracerebrally with plaque-purified clones isolated from yellow fever virus strain 1899/81

There was, however, a marked difference in disseminated infection rates between the Asibi virus strain and its derived large plaque-size vaccine variant (Table 1). Ninety-four percent (31/33) of the mosquitoes were infected with the parent virus, while only 3% (1/32) contracted a disseminated infection with the vaccine virus. The HeLa- passaged (Asibi parent) virus also demonstrated an inability to disseminate and be transmitted by vector mosquitoes (Table 1). However, when this virus was further passaged in mouse brains (2 times) and then fed to mosquitoes, 16% (7/43) developed a disseminated infection to the head and 33% (1/3) transmitted the virus (data not shown); indicating possible reversion to virulence after relaxation of the selection pressure. This strain (HeLa-passage) was also attenuated in monkeys following subcutaneous inoculation (T. P. Monath and A. D. T. Barrett, unpub.).

Table 2. Virulence of yellow fever virus plaque-size variants in 10-day-old mice

Virus Strain & Plaque Size	Route of inoculation		
	Intracerebral*	Intraperitoneal	
	Average survival time (days ± S.E.M.)	$\log_{10}$ PFU/LD_{50}	Average survival time (days ± S.E.M.)
1899/81 Mixed	7.6 (0.9)	3.3	7.8 (0.5)
A2.1 (1) Large	7.0 (0.0)	3.3	7.8 (0.5)
B3.1 Large	6.6 (0.6)	3.7	7.5 (0.6)
B1.1 Medium	7.6 (0.6)	3.7	8.0 (1.0)
A5.1 (1) Small	7.6 (0.9)	3.4	7.0 (1.0)
A3.1 Small	7.0 (0.7)	4.0	7.2 (1.1)
B4.1 Small	7.0 (0.0)	3.4	7.0 (0.7)

* The inoculum was approximately 10,000 PFU.

Mouse virulence

We measured the virulence of the plaque-size variants in 10-day-old mice by average survival time after intracerebral and intraperitoneal inoculation (Table 2, Figure 2) and by determining intracerebral and intraperitoneal LD_{50} (Table 3). As with the mosquito experiments, no significant differences could be demonstrated in mouse virulence by the i.c. and i.p. routes of exposure between the parent virus and the plaque-size variants. The average survival time following i.c. inoculation of 10,000 plaque forming units (PFU) of virus varied from 6.6 days to 7.6 days; i.p. average survival times ranged from 7 to 8 days (Table 2). Intracerebral LD_{50} (10^8 Vero cell PFU/ml) ranged from $10^{8.6}$ to $10^{9.7}$, while i.p. values ranged from $10^{3.9}$ to $10^{5.0}$ (Table 3). Figure 2 depicts mean survival time as a function of virus titer following i.c. inoculation; the curves are all similar to that for the parent virus strain.

Discussion

We compared plaque-size variants of yellow fever virus for their ability to infect and be tansmitted by the epidemic vector, *Ae. aegypti*. The hypothesis we tested was that some genetic variants of yellow fever virus are

Table 3. Susceptibility of 10-day-old mice to plaque-size variants of yellow fever virus

Virus Strain	Plaque Size	Log_{10} LD_5 /ml**		
		Intracerebral	Intraperitoneal	Difference
1989/81 Parent	Mixed	8.9	4.5	4.4
A2.1 (1)	Large	8.6	5.0	3.6
B3.1	Large	9.1	3.9	5.2
B1.1	Medium	9.3	4.3	5.0
A5.1 (1)	Small	9.3	4.6	4.7
A3.1	Small	9.3	4.2	5.1
B4.1	Small	9.7	4.6	5.1

* Small = <1 mm; medium 1–3 mm; large >4 mm; 9 days postinfection in Vero cells.
* All inocula diluted to approximately 10^8 Vero cell PFU/ml; volumes inoculated: i.c., approxi-
* mately 20 microliters, i.p., approximately 100 microliters.

more efficient in their ability to infect and replicate in insect cells. We had hoped to increase our chances of finding these putative variants by examining plaque-purified phenotypic variants. Virulence in vertebrates was also considered because the vaccine virus (17D) is attenuated in mosquitoes as well as in humans and other experimental nammals (Barrett *et al.*, 1987; Deubel *et al.*, 1981). We thought it possible that attenuation of yellow fever virus in vertebrates and in mosquitoes might occur by a common mechanism. Also, since most investigators use plaque-purified material as a source of virus for molecular cloning of genomic nucleic acid and subsequent sequence analysis, we wanted to determine what proportion of a mixed virus population exhibits a wild-type (in a biological sense) phenotype.

We failed to discover any significant differences in the proficiency of the South American plaque-size variant viruses to infect vector mosquitoes or in their virulence for mice. We assume that the virus variants we assayed represent genetic variants; however, the genetic changes in these viruses may have occurred at loci that are not important in vector competence or mouse virulence, and further, loci associated with plaque-size may be irrelevant to these biological attributes. Other workers have demonstrated with 17D virus that plaque-size variants differ in virulence following i.c. inoculation of adult mice and in growth characteristics in human macrophages (Liprandi, 1981; Liprandi and Walder, 1983). However, it appaers that the relative proportion of these virions in the population varies between preparations; this, of course, restates our original hypothesis. Sequence analysis of monoclonal antibody-resistant varients that exhibit attenuated phenotypes in mosquitoes and vertebrates is probably the best approach to understanding which genetic loci are important in the biology of vector competence (Sundin *et al.*, 1987). Another powerful approach is the use of site-directed mutagenesis of infectious DNA clones when they become available.

In a previous study, Barrett *et al.* (1987) reported that *Ae. aegypti* mosquitoes fed the vaccine strain of yellow fever virus developed midgut infections (43/94), however, none of these infections spread to the head. In our study, only one insect (1 of 32) developed a disseminated infection after ingesting 17D virus; this mosquito was unable to transmit the virus. Plainly, this virus (a large plaque-size variant) is attenuated for vactors, even though the virus is quite capable of initially infecting midgut cells. This phenomenon has also been described for a bunyavirus (Miller, 1983). The molecular basis for this restriction in replication is unknown, but might provide investigators a useful tool in attempting to understand how arthropods are able to control arboviral infections.

In a series of experiments conducted between 1963 and 1971, investigators reported that continuous passage of yellow fever virus (Asibi strain) in HeLa cells distinctly reduced peripheral virulence for monkeys (Converse *et al.*, 1971; Hardy, 1963; Heran *et al.*, 1966). In our study, this virus (subjected to the same selection pressure) showed a marked attenuation in vector mosquitoes following oral infection. However, when the virus was grown an additional 2 times in baby mouse brains, one mosquito with a disseminated infection was able to transmit the virus to a suckling mouse. Whether or not the virus transmitted by this vector has reverted to virulence during replication is unknown. It is interesting to note that the 2 virus strains that were restricted in mosquitoes were also attenuated in primates. It is not known if the HeLa cell substrate selects for attenuated variants of other strains of yellow fever virus; the molecular basis of the selection pressure is also not understood.

In summary, plaque size for the South American isolate (1899/81) of yelow fever virus is not a phenotypic marker for virulence in mice or attenuation in vector mosquitoes. The limited number of clonal isolates we examined failed to reveal significant biological differences associated with virulence; a more extensive exploration might divulge variants with altered biological properties. Molecular techniques, including sequence analysis of site-directed attenuated mutants and antigenic characterization with monoclonal antibodies, should be used to study vector competence and virulence of yellow fever virus further.

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Explanation to Figure 1 (Plate XXIX)

Plaque morphology of parental yellow virus fever strains and derived plaque variants. (a) 1899/81 parent; (b) large plaque variant A2.1(1); (c) small plaque variant B4.1; (d) Asibi parent; (e) 17D vaccine strain; (f) HeLa variant.