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Selection for Virulent Dengue Viruses Occurs in Humans and Mosquitoes

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Dengue is the most common mosquito-borne viral disease in humans. The spread of both mosquito vectors and viruses has led to the resurgence of epidemic dengue fever (a self-limited flu-like syndrome) and the emergence of dengue hemorrhagic fever (severe dengue with bleeding abnormalities) in urban centers of the tropics. There are no animal or laboratory models of dengue disease; indirect evidence suggests that dengue viruses differ in virulence, including their pathogenicities for humans and epidemic potential. We developed two assay systems (using human dendritic cells and *Aedes aegypti* mosquitoes) for measuring differences in virus replication that correlate with the potential to cause hemorrhagic dengue and increased virus transmission. Infection and growth experiments showed that dengue serotype 2 viruses causing dengue hemorrhagic fever epidemics (Southeast Asian genotype) can outcompete viruses that cause dengue fever only (American genotype). This fact implies that Southeast Asian genotype viruses will continue to displace other viruses, causing more hemorrhagic dengue epidemics.

Dengue virus pathogenesis has been difficult to examine because there are no laboratory or animal models of the disease. There are four distinct antigenic types (serotypes 1 through 4) of dengue virus (of the *Flaviviridae* family, with a single-stranded, positive-sense RNA genome), and infection by more than one type increases the risk of developing dengue hemorrhagic fever (DHF), a fatal form of dengue (31). Both the infecting viral strain (8, 9, 23) and host immune status (11, 25) influence the severity of dengue, but their relative contributions cannot be directly measured; this fact is important to consider when preparing single or tetravalent vaccines that will not induce DHF (12). However, phylogenetic (17, 21, 22) and epidemiologic (18, 27, 30) studies have pointed to specific viral genetic types (genotypes) as being capable of producing DHF epidemics in a population base of variable immune status. For example, in the Western Hemisphere, the origin and spread of DHF can be linked to viruses of the Southeast Asian genotype, whereas American genotype viruses have been isolated solely from patients presenting with dengue fever (DF); this suggests important biological differences among viral genotypes. Here, we describe two assay systems, using primary human dendritic cells (DCs) (3) and whole mosquitoes (1, 2), for measuring the ability of wild-type dengue viruses to infect and replicate and to presumably mimic their pathogenesis and transmission in human populations. The strikingly increased output of some of these viruses over others in both systems suggests that viruses with the potential to cause DHF (Southeast Asian genotype) have a selective advantage over their more benign counterparts (American genotype) by generating elevated viremias in the human host and infecting a greater proportion of vector mosquitoes. Earlier observations in the South Pacific and Southeast Asia suggested that less virulent dengue virus strains attain lower viremias in the human host, thereby infecting fewer mosquitoes at lower transmission rates (8, 9). Our results provide evidence for a natural viral evolution towards virulence, which can explain an epidemic process.

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MATERIALS AND METHODS

Dengue viruses and phylogeny. Twenty-four dengue serotype 2 virus isolates were used during the course of this study (Table 1). All had undergone limited in vitro passage in mosquito cell lines (five passages or less) to reduce mutations that might be associated with tissue culture adaptation. These isolates were first determined to belong to serotype 2 by an indirect fluorescent antibody (IFA) test with serotype-specific monoclonal antibodies; then the nucleotides encoding the envelope glycoprotein, E, were amplified by reverse transcription (RT)-PCR and sequenced using methods described previously (14). Nucleotide sequences were stored, edited, and aligned by using the Lasergene software package (DNASTAR, Madison, Wis.). The phylogenetic tree presented here was generated by maximum likelihood analysis of the 1,485 nucleotides encoding the E gene (for all samples except the serotype 3 representative, which had 1,379 nucleotides), and bootstrap values of statistical support for branches were calculated by 100 reiterations with the PAUP* software package (26).

DC infections. Conditions similar to those described previously were used to culture monocyte-derived DCs (3). Peripheral blood mononuclear cells were isolated from buffy coats prepared from 1 pt of blood (South Texas Region Blood and Tissue Center, San Antonio) by centrifugation onto a cushion of Histopaque-1077 (Sigma, St. Louis, Mo.). Monocytes were purified (>90%) with a monocyte negative-selection kit according to the manufacturer's instructions (Dynal Inc., Lake Success, N.Y.). Cells were counted and placed in 24-well plates at densities of 1×10^5 to 3×10^5 cells per well. Every other day, one-half of the medium was replaced with medium containing double the concentration of fresh cytokines; cultures were maintained under these conditions for 6 days prior to infection. For most viruses, cells were infected at a multiplicity of infection (MOI) of 1,000 genome equivalents per cell; lower MOIs, of 300 to 500 genome equivalents per cell, were used for viruses 132 and 328298 because of low-titer stocks. The lower MOIs did not adversely affect infection, since the percentages of infected cells for viruses 132 and 328298 were similar to those for other viruses used in each experiment. Extracellular media for quantitative RT-PCR was prepared by centrifuging all samples at $800 \times g$ for 2 min to pellet cells and collecting the resulting supernatant. A portion of the cells was washed in phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PBS−) and placed in Trizol (Invitrogen, Rockville, Md.) for extraction of intracellular RNA. The majority of the cells were prepared for immunostaining and flow cytometry. All RNAs for

TABLE 1. Dengue serotype 2 viruses used in this study

Strain	Genotype[a]	Passage history[b]	Location	Yr	Clinical diagnosis
780447	AM	C6/36 p3	Trinidad	1978	DF
Ven2	AM	AP61 p2, C6/36 p3	Maracay, Venezuela	1987	DF
131	AM	C6/36 p3	Sonora, Mexico	1992	DF
132	AM	C6/36 p2	Sonora, Mexico	1992	DF
328298	AM	C6/36 p3	Reynosa, Mexico	1995	DF
IQT2133	AM	C6/36 p4	Iquitos, Peru	1996	DF
IQT2913	AM	C6/36 p4	Iquitos, Peru	1996	DF
Mara3	SEA	C6/36 p3	Maracay, Venezuela	1990	DF
39325	SEA	C6/36 p3	Rio de Janeiro, Brazil	1990	DF
102954	SEA	C6/36 p4	Aragua, Venezuela	1991	DF
360236	SEA	C6/36 p2	Bucaramanga, Colombia	1992	DF
360281	SEA	C6/36 p2	Tolima, Colombia	1992	DF
CO257	SEA	C6/36 p4	Bangkok, Thailand	1994	DF
CO385	SEA	C6/36 p2	Bangkok, Thailand	1994	DHF
CO576	SEA	C6/36 p2	Bangkok, Thailand	1994	DHF
K0005	SEA	C6/36 p2	Kamphaeng Phet, Thailand	1994	DHF
K0049	SEA	C6/36 p2	Kamphaeng Phet, Thailand	1995	DHF
49255	SEA	C6/36 p4	Rio de Janeiro, Brazil	1995	DF
15957	SEA	C6/36 p4	Maracay, Venezuela	1996	DHF
19966	SEA	C6/36 p4	Maracay, Venezuela	1996	DF
CO168	SEA	C6/36 p2	Bangkok, Thailand	1996	DHF
CO489	SEA	C6/36 p4	Bangkok, Thailand	1996	DF
124B	SEA	C6/36 p2	Santa Cruz, Bolivia	1998	DF
Oax468	SEA	C6/36 p2	Oaxaca, Mexico	2000	DF

[a] American (AM) or Southeast Asian (SEA) genotype classification according to Rico-Hesse (21) and Fig. 1.
[b] Mosquito cell line is followed by number of passages (e.g., p3, three passages).

quantitative RT-PCR were prepared by extraction with Trizol as previously described (3).

Immunostaining and flow cytometry. Cells were washed one time in fluorescence-activated cell sorter (FACS) staining buffer (PBS− containing 1% fetal calf serum) prior to examination for intracellular dengue virus antigen by use of the CytoFix/CytoPerm kit (BD Biosciences Pharmingen, San Diego, Calif.). Fluorescein isothiocyanate (FITC)-labeled anti-dengue virus monoclonal antibody was used to detect intracellular viral protein, following procedures previously described (3). DCs were washed twice with CytoPerm/CytoWash buffer to remove unbound antibody, and cells were fixed in PBS− containing 1% fetal calf serum and 4% paraformaldehyde. All samples were acquired and analyzed with a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, N.J.) within 24 h of staining.

Quantitative, real-time RT-PCR. (i) Single-step reactions. The conditions used for the quantitative RT-PCR were described previously (1). This system was used to determine the number of dengue virus genome equivalents in infected cell culture supernatants and to standardize virus quantities in mosquito blood meals.

(ii) Two-step reactions. A two-step assay was employed to detect intracellular positive- and negative-strand dengue virus RNAs. Conditions similar to those described for the one-step reaction were used, with the following modifications. RT was performed either for positive-strand RNA with primer PA-4 or for negative-strand RNA with primer PA-1. Five microliters of Trizol-extracted intracellular RNA or in vitro-transcribed strand-specific control RNA was heated at 90°C for 10 min and quick-chilled on ice for denaturation. Ten microliters of the RT reaction mix was added to each reaction mixture, resulting in final concentrations of 1× core RT-PCR buffer, 5.5 mM $MgCl_2$, a 0.8 mM total concentration of deoxynucleotides, and 166 nM strand-specific primer, and 0.5 U of RNase inhibitor and 1.0 U of StrataScript RT were added last. Reaction mixtures were incubated for 20 min at 50°C, followed by the inactivation of RT by heating to 99°C for 10 min. One-third of each RT reaction mixture was transferred to a new tube containing 45 μl of PCR mix. Optimal conditions for negative-strand PCR involved the same concentrations of salts, enzymes, probes, and primers as described for the single-step reaction; 200 nM concentrations of PA-1 and PA-4 resulted in more efficient PCR amplification for positive-strand RNA. Negative controls included positive- and negative-strand RNA reverse transcribed in the presence of the wrong primer.

Mosquito infections. *Aedes aegypti* females, from an F_1 generation of a colony collected from McAllen, Tex., were reared in the laboratory and then allowed to cofeed on two virus strains by using a water-jacketed membrane feeder. Each blood meal contained representatives of the Southeast Asian and American genotypes at equal concentrations of 2.5×10^8 genome equivalents/ml in EDTA-treated rabbit blood. Mosquito heads were removed and assayed for the presence of dengue virus antigen by the IFA test (13).

Genotype-specific RT-PCR. Mosquito legs were removed, and their RNA was extracted in 0.5 ml of Trizol according to the manufacturer's specifications, except that 10 μg of glycogen was added during the precipitation step. RNA pellets were diluted in 20 μl of H_2O, and 6 μl of this preparation was added to a 22-μl RT reaction mixture containing 15 mM Tris-HCl (pH 8), 50 mM KCl, 5 mM $MgCl_2$, a 1.0 mM total concentration of four deoxynucleotides, a 1.1 μM concentration of random nanomers, 25 U of reverse transcriptase, and 10 U of RNase inhibitor (Applied Biosystems, Foster City, Calif.). RT reaction mixtures were incubated at 25°C for 5 min, 42°C for 15 min, and 99°C for 5 min. Ten microliters of RT reaction mixtures was added to two PCRs containing primers targeting either the Southeast Asian genotype (Den2-Asn, 5′-GCCTTGTTTTATGTTAGTTCTGC-3′; Den2-C, 5′-CAGTGGAATCATGGGAGGAAATC-3′) or the American genotype (Den2-Am, 5′-CTTTTGTTCTGTCTCATGTTTTTCT-3′; Den2-G, 5′-GATGACGACGGAAGACATGTTGACAGTT-3′). PCR mixtures contained 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, a 1.0 mM total concentration of four deoxynucleotides, 1.25 U of AmpliTaq Gold DNA polymerase, 500 nM Den2-Asn or Den2-Am, and 500 nM Den2-C or Den2-G. Amplification was performed as follows: 10 min at 95°C (1 cycle); 30 s at 94°C, 30 s at 60°C for primers Den2-Asn and Den2-C or 30 s at 62°C for primers Den2-Am and Den2-G, and 30 s at 72°C (35 cycles); and 5 min at 72°C (1 cycle). Amplification products were separated on 2% agarose gels with expected sizes of 243 and 318 bp for the Southeast Asian and American genotypes, respectively.

Statistical analyses. Data were analyzed with InStat version 3.00 software (GraphPad Software, San Diego, Calif.). Infection rates and virus outputs were pooled for each genotype (American versus Southeast Asian) or for each clinical association (DF versus DHF), and these values were compared by unpaired *t* tests when the conditions of normality were valid. Data sets that deviated from a normal distribution by the Kolmogorov-Smirnov test were instead analyzed by the Mann-Whitney U test. Mosquito infection rates were compared by analysis of variance to account for variation within and between genotypes; the chi-square test was used to determine the probability of dual infection by more than one genotype in direct competition experiments.

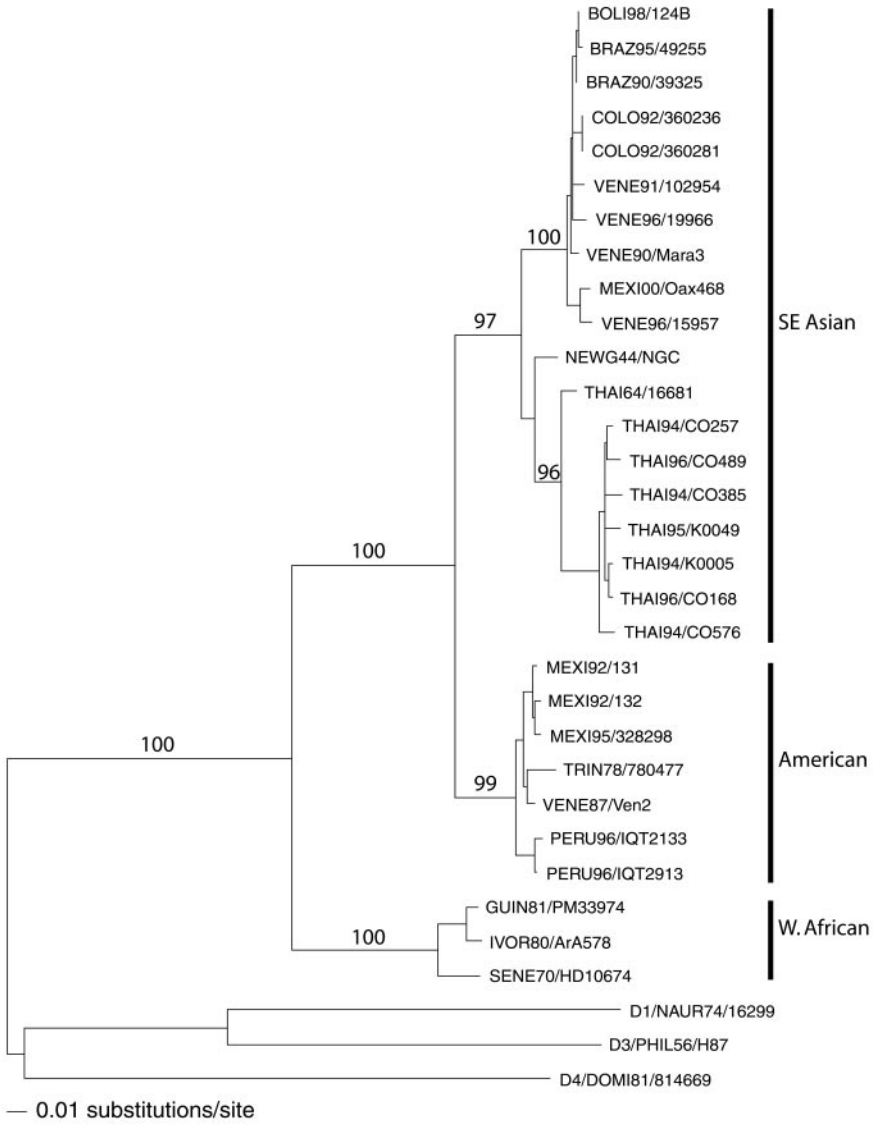

FIG. 1. Phylogeny of serotype 2 dengue viruses. The 24 viruses used in this study are shown in comparison to 3 viruses from western Africa, used as an outgroup since they are the most divergent, and representatives of the other three serotypes, used to root the tree. Strains are identified by country of isolation, year of isolation, and strain number (Table 1). Genotypic groups are marked by vertical lines, with names given to the right. Bootstrap values of statistical support for major branches are given as percentage equivalents. The horizontal distance between strains represents the extent of genetic divergence (see scale).

Nucleotide sequence accession numbers. The GenBank accession numbers for the various viruses are as follows: for strain D1/16299, accession number M23027; D3/H87, M93130; D4/814669, AF326573; HD10674, AF231720; ArA578, AF231718; PM33974, AF231719; IQT2913, AY158339; IQT2133, AY577439; Ven2, AY158328; 780477, AY158327; 328298, AY158338; 132, AY158333; 131, AY158332; CO576, AY158335; CO489, AY577438; CO257, AY577437; CO168, AY577436; CO167, AY577435; K0005, AY158336; K0049, AY158337; CO385, AY158334; 16681, U87411; NGC, D00346; 15957, AY577434; Oax468, AY158341; Mara3, AY158329; 19966, AY577433; 102954, AY158330; 102091, AY577432; 360281, AY577431; 360236, AY158331; 49255, AY577430; and 124B, AY158340.

RESULTS

Phylogenetic analysis of the 24 dengue virus serotype 2 isolates used for comparison was performed with the 1,485 nucleotides encoding E, and as in previous studies, these viruses clustered as two distinct groups based on their evolutionary origins (Fig. 1). All seven American genotype viruses were isolated in Mexico and South America between 1978 and 1996. Of the 17 Southeast Asian genotype viruses, 10 were isolated in Mexico and South America and 7 were isolated in Thailand, all between 1990 and 2000. Two serotype 2 reference strains, New Guinea C and 16681, were also included in the phylogenetic analysis; these viruses were isolated much earlier (1944 and 1964, respectively) and had undergone very high passage in cell culture and other organisms (monkeys, mice, and/or mosquitoes). Previous studies had shown that viruses belonging to the Southeast Asian genotype have produced DHF, while those belonging to the American genotype have produced DF only, in spite of prior immunity (i.e., secondary infections) (22, 30). The Southeast Asian viruses are not new and have evolved

little over a 50-year period (e.g., New Guinea samples from 1944 versus Thai samples from 1994), yet they have effectively spread to the American continents and Pacific islands in the past 20 years, apparently displacing the indigenous serotype 2 viruses characteristic of those areas (Fig. 1). That is, most isolations of serotype 2 virus from patients in the Western Hemisphere and Pacific Islands are of the Southeast Asian genotype, and the American genotype viruses are harder to detect (last reported only in northern Mexico and Peru).

To determine whether dengue virus genotypes differ in their abilities to infect the known primary target cells in humans (16, 32), DC cultures from five anonymous blood donors were infected with 7 dengue viruses of the American genotype (780447, Ven2, 131, 132, 328298, IQT2913, and IQT2133) and 12 viruses of the Southeast Asian genotype (Mara3, 39325, 102954, 360236, 360281, CO385, CO576, K0005, K0049, 49255, 124B, and Oax468). The percentage of infected cells was estimated by FACS analysis using an FITC-labeled anti-dengue virus antibody. As a group, the American genotype viruses infected significantly more DCs than the Southeast Asian genotype viruses did ($P < 0.0001$), according to data pooled for the five donors (Fig. 2a). However, the magnitude of this difference was relatively minor (mean of 12% DCs infected by American genotype viruses versus 8% infected by Southeast Asian genotype viruses), and this trend did not hold for all of the blood donors tested (Fig. 2a). The percentages of infected DCs varied markedly among different blood donors, ranging from 4 to 16% and 2 to 15% for the American and Southeast Asian genotypes, respectively.

To compare the efficiencies at which these genotypes replicate in target cells, quantitative RT-PCR and FACS analyses were used to estimate genomic equivalents secreted per infected DC, which is referred to here as virus output. The Southeast Asian genotype viruses consistently generated higher virus outputs in DCs than the American genotype group for all of the blood donors (Fig. 2b). Overall, virus output for Southeast Asian genotype viruses was about fivefold greater than that observed for American genotype viruses ($P < 0.0001$), according to the data pooled for the five donors.

Since similar infection rates were observed, the disparity in dengue virus output between the two genotypes may reflect differences in the efficiency of positive- and/or negative-strand synthesis during the viral RNA replicative cycle. To explore this possibility, virus output was measured at 48 h postinfection and compared to the accumulation of intracellular negative- and positive-strand viral RNA. Three American genotype viruses (Ven2, 131, and IQT2913) and six Southeast Asian genotype viruses (15957, 19966, CO257, K0005, CO168, and CO489) were selected for this comparison. In confirmation of prior results, the Southeast Asian genotype viruses consistently generated higher virus outputs than the American genotype viruses ($P < 0.01$) (Fig. 3a). In addition, the Southeast Asian viruses generated greater amounts of negative-strand and positive-strand RNA per infected cell than the American viruses did at all time points (Fig. 3c and d). Differences in negative- and positive-strand accumulation tended to be greatest during the early time points of 4, 8, and 12 h postinfection (P, <0.1 to <0.0001). We also reanalyzed the data on the basis of clinical association, comparing DF isolates (Ven2, 131, 19966, IQT2913, CO257, and CO489) to DHF isolates (15957, K0005, and CO168). Virus output from the DHF isolates was about twofold greater than that observed from DF isolates, although this difference was not statistically significant (Fig. 3b).

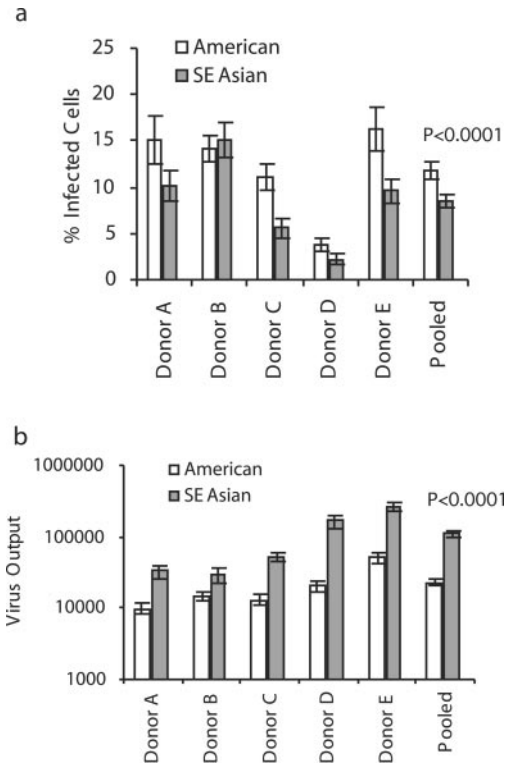

FIG. 2. Dengue virus infectivity and output in DCs. (a) Infected DC cultures were examined by FACS 48 h postinfection with an FITC-labeled anti-dengue virus-specific antibody to estimate the percentage of infected cells. (b) Virus output was estimated by dividing the number of genome equivalents in culture supernatants (by quantitative RT-PCR assay) by the number of infected cells. Graphs were generated from data for individual donors (A through E) and from pooled data for all donors. Within each graph, the white bars represent the mean results for 7 American genotype viruses (780447, Ven2, 131, 132, 328298, IQT2913, and IQT2133) and the gray bars represent the mean results for 12 Southeast Asian genotype viruses (Mara3, 39325, 102954, 360236, 360281, CO385, CO576, K0005, K0049, 49255, 124B, and Oax468). Error bars represent the standard errors of the means, with infections performed in triplicate.

Differences in virus output suggest that Southeast Asian genotype viruses have a selective advantage over the American genotype viruses by generating higher viremias in the human host. To explore whether the main mosquito vector (*A. aegypti*) of dengue virus may also select for one particular genotype, we cofed mosquitoes competing viral strains representing the Southeast Asian and American genotypes at equal titers. Mosquitoes were subsequently held for 14 days at 30°C and assayed for disseminated infection by IFA testing of head tissue and genotype-specific RT-PCR of the legs. Dengue viruses representing the Southeast Asian genotype infected and disseminated in mosquitoes more efficiently than did viruses of the American genotype in direct competition experiments (Table 2). Of 270 exposed mosquitoes (F_1 generation), 21% became infected solely with the Southeast Asian genotype versus 3% with the American genotype only ($P < 0.001$). Mosquitoes were more likely to become coinfected by both genotypes (3%)

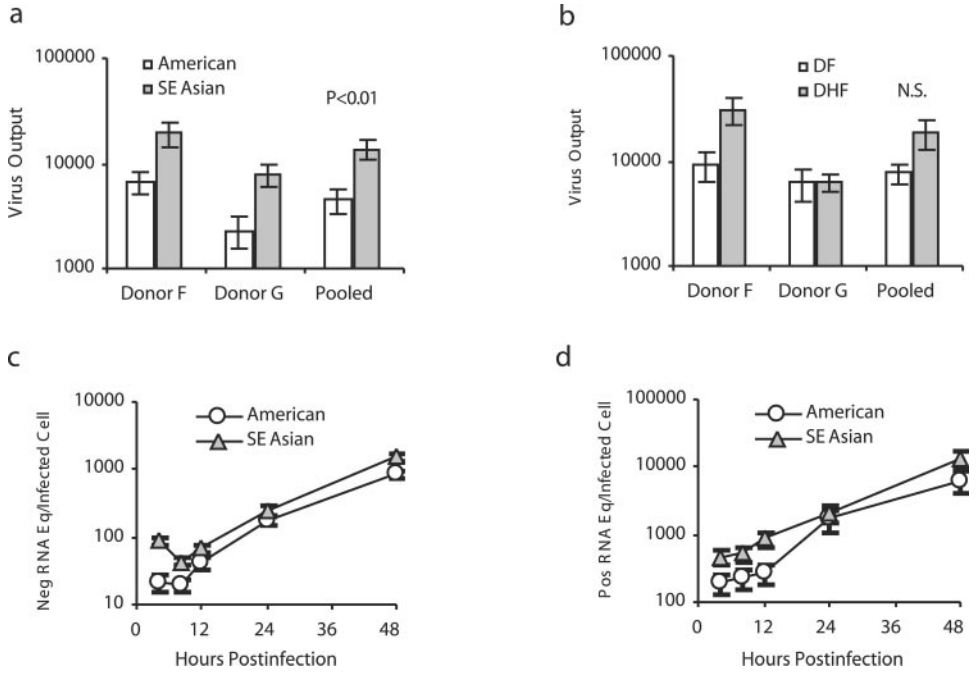

FIG. 3. Dengue virus output and RNA replication in DCs. (a and b) Graphs depicting the mean virus outputs at 48 h postinfection, as described in the legend to Fig. 2b. The graphs compare American (white bars) and Southeast Asian (gray bars) virus genotypes (a) and virus clinical associations for DF (white bars) and DHF (gray bars) (b). (c and d) Quantitative RT-PCR and FACS analyses were done with the same samples as those used for panel a to estimate intracellular negative-strand and positive-strand RNAs at various times postinfection. *P* values from unpaired *t* tests were as follows: for intracellular negative-strand RNA (Neg RNA Eq), <0.0001 at 4 h, 0.0012 at 8 h, 0.0461 at 12 h, 0.2127 at 24 h, and 0.0144 at 48 h (c); for intracellular positive-strand RNA (Pos RNA Eq), 0.027 at 4 h, 0.0615 at 8 h, 0.0186 at 12 h, 0.68 at 24 h, and 0.0984 at 48 h (d). Mean data points for American genotypes are represented by open circles, and mean data points for Southeast Asian genotypes are represented by gray triangles. Error bars represent the standard errors of the means, with infections performed in triplicate. N.S., not significant.

than would be expected by chance ($P < 0.05$), suggesting that viral genotypes compete to infect the same individuals within a mosquito population.

DISCUSSION

Taken together, these results suggest that the Southeast Asian viruses have an early replicative advantage in the human host and infect a greater number of mosquitoes, thus allowing them to spread more effectively than their American counterparts. This difference in transmission and subsequent spread had been documented for other dengue virus genotypes (20), but the Southeast Asian genotype remains the only one directly linked to severe disease and displacement of another genotype. Since higher viremias have been associated with DHF (15, 19, 28, 29), these results support the view that Southeast Asian viruses are more pathogenic and that more efficient virus replication in the primary target cells (the immature DCs which

TABLE 2. Ability of competing dengue virus strains representing Southeast Asian and American genotypes to infect mosquitoes

Blood meal		No. of mosquitoes tested	% of mosquitoes positive for genotypes by IFA test	% of mosquitoes positive by RT-PCR for indicated genotype		
Southeast Asian	American			Southeast Asian	American	Both
K0049	131	32	15.6	12.5	0.0	0.0
	Ven2	29	20.7	13.8	6.9	0.0
	IQT2913	29	27.6	17.2	6.9	3.4
CO385	131	28	25.0	21.4	0.0	3.6
	Ven2	38	23.7	21.1	0.0	2.6
	IQT2913	23	39.1	34.8	0.0	4.3
102954	131	31	35.5	16.1	9.7	3.2
	Ven2	30	26.7	10.0	6.7	10.0
	IQT2913	30	46.7	46.7	0.0	0.0
Total		270	28.5	21.1	3.3	3.0

reside in the epidermis) provides a selective advantage over viruses of lower pathogenic potential. In the mosquito, infection proceeds through a large variety of cells and organs; our test for dissemination measures the end result of selection in this complex milieu but reflects the virus most probable to be transmitted to other humans. Our results also indicate that one mechanism possibly involved in determining higher virus output is the efficiency at which negative-strand RNA is generated early during infection. This generation would result in an increase in genomic RNA, which would increase the number of secreted infectious viral particles; thus, the Southeast Asian viruses are better adapted for production of virus progeny, independent of the number of cells they infect. When considering this direct effect in vivo, one can see that it explains the abilities of Southeast Asian viruses to replicate in the human body and infect the mosquito, thus initiating the cascade of events responsible for DHF in humans, and to be transmitted at much higher rates than the American counterpart in mosquitoes.

When viruses from DF patients were compared to those from patients meeting the DHF case definition, there was no significant difference in the amount of virus produced, but outputs varied from one donor to another, possibly reflecting individual variation in response to infection and, thus, clinical presentation. That is, the Southeast Asian genotype viruses have the potential to produce much more virus in some individuals, thus producing a higher viremia and, concomitantly, more severe disease. The prior immune status of the individual (humoral and cellular immunity to other dengue viruses) most probably increases the viremia and immunopathogenesis further, as described previously (10, 24). These increases would explain the different host responses during epidemics and reflect the complexity of DHF pathogenesis but bode ill for the formulation of standardized vaccines and the hopes of establishing an animal model of DHF.

The integration of both higher viremias for humans and higher transmission rates for mosquitoes would have a significant impact on the epidemiology of disease; the two factors we measured are probably very important mechanisms of selection for viruses with higher virulence in nature. In terms of evolutionary theory, our data suggest that dengue viruses do not trade off fitness by becoming better adapted at replicating in both humans and mosquitoes (4, 5). We found no evidence for rapid genetic divergence in the genotypic groups we compared, and the main mechanism for change seems to be point mutation and selection. The overall result of selection to form the Southeast Asian genotype is that it has gradually become much more fit than other genotypes and is expanding geographically to occupy other niches. It remains to be seen if this genotype can now displace the two genotypes found in Africa (sylvatic and imported epidemic strains), although the vectors there may differ in susceptibility to dengue virus infection (6, 7); this displacement would have a severe impact on the international public health infrastructure, which is already burdened by over 50 million dengue virus infections per year.

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