Differential Evolution of Eastern Equine Encephalitis Virus Populations in Response to Host Cell Type

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ABSTRACT

Arthropod-borne viruses (arboviruses) cycle between hosts in two widely separated taxonomic groups, vertebrate amplifying hosts and invertebrate vectors, both of which may separately or in concert shape the course of arbovirus evolution. To elucidate the selective pressures associated with virus replication within each portion of this two-host life cycle, the effects of host type on the growth characteristics of the New World alphavirus, eastern equine encephalitis (EEE) virus, were investigated. Multiple lineages of an ancestral EEE virus stock were repeatedly transferred through either mosquito or avian cells or in alternating passages between these two cell types. When assayed in both cell types, derived single host lineages exhibited significant differences in infectivity, growth pattern, plaque morphology, and total virus yield, demonstrating that this virus is capable of host-specific evolution. Virus lineages grown in alternation between the two cell types expressed intermediate phenotypes consistent with dual adaptation to both cellular environments. Both insect-adapted and alternated lineages greatly increased in their ability to infect insect cells. These results indicate that different selective pressures exist for virus replication within each portion of the two-host life cycle, and that alternation of hosts selects for virus populations well adapted for replication in both host systems.

As a group, the arthropod-borne viruses (arboviruses) include many important human and animal pathogens. In nature arboviruses are routinely maintained by transmission cycles involving the passage of virus between susceptible vertebrate hosts and hematophagous arthropod vectors. By definition arboviruses are spread by biological transmission and must be able to replicate within both vertebrate and invertebrate hosts (Chamberlain and Sudia 1961; Turell 1988).

Eastern equine encephalomyelitis virus (EEE; family Togaviridae, genus Alphavirus) is an arbovirus that is endemic to the eastern half of North America, Central America, and South America. In the United States, it is maintained in a sylvatic transmission cycle involving a variety of passerine bird species and the enzoonotic mosquito vector, *Culiseta melanura* (Scott and Weaver 1989). Occasionally other vertebrates are infected. The virus is particularly virulent for humans, horses, and gamebirds (Scott *et al.* 1994).

Despite the potentially high mutation rate of its RNA genome (Steinhauer and Holland 1987), multiple studies have described a high degree of antigenic conservation and slow rates of molecular evolution for the EEE virus (Roehrig *et al.* 1990; Weaver *et al.* 1991, 1992, 1993a,b; Strizki and Repik 1994; Weaver 1995). The stability of the EEE virus in nature is consistent with the observation that, in general, arbovirus populations tend to be genetically conserved over both time and space (Beaty *et al.* 1988; Weaver *et al.* 1992; Scott *et al.* 1994; Cilnis *et al.* 1996; Mackenzie *et al.* 1996; Poidinger *et al.* 1997). The constraints associated with a two-host life cycle may favor the maintenance of specific viral genotypes (Scott *et al.* 1994; Weaver *et al.* 1999) and has often been suggested as the underlying mechanism for the observed stability of arbovirus genomes.

Previous studies with other Togavirus systems indicate that both phenotypic and genotypic characteristics can be influenced by host cell type and that differential selection for host-specific mutants is possible (Kowal and Stollar 1981). Host-associated limitations on growth, virion biochemistry, plaque morphology, temperature sensitivity, and infectivity have been documented (Gliedman *et al.* 1975; Renz and Brown 1976; Luukkonen *et al.* 1977; Symington and Schlesinger 1978; Kowal and Stollar 1981; Durbin and Stollar 1984, 1986; Brown and Condreay 1986; Strauss and Strauss 1994; Heidner *et al.* 1996). Thus, virus infection and replication are expected to be dissimilar within vertebrate and arthropod hosts. This led to the suggestion that the presence of host-specific selective pressures limit the rate of evolution by imposing a fitness tradeoff; that is, optimization to one host decreases viral fitness in the alternate host (Strauss and Strauss 1994; Novella *et al.* 1995). An alternative explanation for the lack of expected variation in arbovirus populations is

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that, through time, arboviruses have evolved to maximize replication in both vertebrates and invertebrates by simultaneously adapting to the dual selective pressures associated with obligate cycling between two different kinds of hosts. Under such a model, virus populations that are continually cycled between two hosts grow well and maintain high fitness in both systems.

Our long-term research objective is to examine the role of different hosts in arbovirus evolution using an alphavirus study system to more fully understand the relationships of alphaviruses and perhaps arboviruses in general to their vertebrate and invertebrate hosts. Toward that end, we conceived and initiated a series of experiments. The first, described herein, was to use a cell culture system to generate derived virus lineages that had been repeatedly transferred through either vertebrate or invertebrate cells or in alternating passages between these two cell types. In subsequent experiments we repeated this design using intact animal hosts. Once generated and stored in a frozen state, these lineages could then be examined for host-specific adaptations by various means or passaged further. For instance, viral fitness assays could be performed to determine the presence or absence of fitness costs under the trade-off model using the previously generated virus lineages. Ultimately, individual virus lineages could be molecularly characterized to determine the genetic correlates of observed host-specific phenotypic changes.

In this article, we describe the first of these experiments in which we compared the growth characteristics of multiple EEE virus lineages adapted to replication in different host cell types using standard virological methods. Our study consisted of two parts. First, we determined whether the presence of different selective pressures associated with replication within a single cell type, vertebrate or invertebrate, results in divergent virus populations. Second, we determined whether virus populations transferred between vertebrate and invertebrate cells evolve to display growth characteristics common to virus populations adapted to either of the single cell types. Upon entering into these studies, we did not attempt to predict the direction or nature of virus adaptation. Rather, we sought to describe phenotypic changes by using standard virological and statistical analyses and to interpret the evolutionary significance of these results within the context of current alphavirus literature.

MATERIALS AND METHODS

Media and cell lines: All cell lines were maintained in minimum essential medium (Eagle) with Earle's balanced salt solution supplemented with 10% fetal calf serum. Routine cell maintenance was carried out in a 5% CO_2 atmosphere at ambient temperatures of 37° and 28° for vertebrate and insect cells, respectively.

Mammalian cells: Subclone 21 of the baby hamster kidney cell line (BHK-21) was used for production and quantification of all viral stocks (Scott and Burrage 1984).

Avian cells: A Peking duck embryo (PDE) cell line was obtained (American Type Culture Collection, Rockville, MD) at the ninth passage and subcultured for up to 18 passages thereafter. This cell line has previously been shown to support the replication of EEE virus to high titers and displays cytopathic effects (Marcovici and Prier 1968).

Mosquito cells: The C7-10 cell line was obtained (V. Stollar, Rutgers University) at the second passage and continuously subcultured thereafter. This subclone of the C6/36 larval *Aedes albopictus* cell line (Igarashi 1978) is unusual among arthropod cell cultures in that it displays marked cytopathic effects following infection by the EEE virus (Sarver and Stollar 1977).

EEE virus stock: For all of our experiments we used a low passage North American EEE virus; strain 82V-2137 isolated in 1982 from a pool of naturally infected Florida mosquitoes (N. Karabatsos, Centers for Disease Control and Prevention, Fort Collins, CO). The strain was originally isolated in primary duck embryo cells and then passaged twice in African green monkey kidney (Vero) cells. In addition to its known passage history, an additional advantage of using this virus is that its entire nucleotide sequence is known (Chang and Trent 1987; Weaver *et al.* 1993b). For our studies, we generated a plaque-purified stock virus on BHK-21 cells and stored multiple aliquots at −70°. This ancestral stock was used as the starting virus population from which all subsequent lineages were derived and served as the control population against which derived virus lineages were compared.

Virus quantification: The total number of virions present was estimated in BHK-21 cells either by 50% tissue culture infectious dose ($TCID_{50}$; Reed and Muench 1938) or by duplicate plaque assay in six-well plates (Cooper *et al.* 2000). Following a 48-hr incubation period, cells were examined for cytopathic effect or plaques, respectively.

Statistical procedures: Parametric statistics were used when the assumptions of normality and homogeneity of variances were met or could be satisfied by an appropriate data transformation. Data sets that did not satisfy these assumptions were analyzed by nonparametric procedures. Probability (P) values equal to or less than 0.05 were considered to be biologically significant. Mean comparison procedures were employed only after significance differences were detected by an overall analysis of variance (ANOVA). Means were compared by the least significant differences test (LSD; Sokal and Rohlf 1981). Mean data are most often displayed as the arithmetic mean ± the standard error of the mean (SEM).

Selective passages: Thirty virus lineages were derived from a single vial of the original virus clone. Ten lineages were serially propagated in mosquito cells, 10 in avian cells, and 10 were alternated between these two cell types. The alternated lineages were first passed through insect cells, which resulted in their final passage being through the avian cell line. Serial passages were carried out in six-well tissue culture plates containing confluent cell monolayers covered by 4 ml of liquid media. The number of cells per well averaged between 10^5 and 10^7. Virus amplification was allowed to proceed for 48 hr and was confirmed by the presence of cytopathic effects. An aliquot of each lineage was harvested, quantified as $TCID_{50}$/ml on BHK-21 cells, and frozen at −70°. A total of 10 selective passages were performed for each treatment group. To minimize the effects of defective interfering particles, the $TCID_{50}$ value from the previous passage was used to dilute each lineage to ensure that the multiplicity of infection (MOI) was ~0.1 for each round of virus amplification. By maintaining a constant MOI for each lineage at each passage, we ensured that the virion to cell ratio was always equal among the three

treatment groups, regardless of each lineage's starting titer. A consistent MOI also allowed us to maintain a large population size of between 10^4 and 10^6 virions at each selective passage. All selective passages and assays were carried out at 34.5° to avoid the unintended selection of temperature-sensitive mutants. Time-dependent changes in virus growth patterns were assessed after the tenth passage by a factorial ANOVA using the BHK-21 cell $TCID_{50}$ values calculated for all 30 lineages at each of the 10 passages (Steele and Torrie 1980).

Relative detection of viruses in different cell types: Because we examined the effects of cell-specific virus adaptation, our ability to accurately quantify the number of virions present in any given sample was essential. Preliminary testing for cell-specific changes in virus infectivity was accomplished by determining virus titers as $TCID_{50}$ for each of the 30 derived virus lineages and the ancestral control in each of three cell types, insect (C7-10), avian (PDE), and mammalian (BHK-21). Following the tenth selective passage, a single aliquot of each lineage was simultaneously quantified (in triplicate) on each of the three cell types. This allowed detection of host range mutants (*i.e.*, viruses not capable of growth in certain cell types), as well as a determination of the susceptibility of each cell line to viruses that had undergone different selective regimes. The mean $TCID_{50}$ values of each lineage for each cell type were calculated, analyzed by ANOVA, and compared by the LSD procedure (Sokal and Rohlf 1981). Data were rank transformed prior to statistical analysis.

Plaque morphology and size: Altered plaque appearance was used as an initial indicator of lineage divergence. When grown on BHK-21 cells, the clonal ancestral population was made up of virions that caused uniformly large round plaques. Therefore, to assess if selective passages had resulted in the emergence of different virus populations the appearance and relative size of each lineage's viral plaques were recorded following plaque assays on BHK-21 monolayers. This initial screening subjectively determined the number of distinct plaque phenotypes within each derived lineage. Plaque phenotype determinations were made based on relative plaque size and several general morphological features, which included the following. The overall shape of the plaque was scored as either round or star-like, the former being symmetrical with a uniformly smooth perimeter and the latter asymmetrical with irregular borders. For plaque size determinations, only well-isolated plaques were measured. Size was measured as maximum plaque diameter using a dissecting stereo microscope fitted with an ocular micrometer. To control for daily variations, ancestral virus was concurrently assayed and used as a standard population against which each experimental lineage was compared. Values are expressed as a percentage of the control value. Up to 50 plaques were examined for each lineage and its corresponding ancestral control. Significant mean differences between individual lineages and the ancestral control, as well as those between passage groups, were determined by the Kruskal-Wallis statistic using the 50 individual plaque measurements from each of the 10 lineages. Significant differences between treatment groups were determined by an ANOVA followed by the LSD means procedure (Sokal and Rohlf 1987).

Virus yields: Fecundity was measured as the level of virus production at the end of a 48-hr growth period. After the tenth selective passage aliquots of each of the 30 derived lineages and the ancestral control were diluted, as in the selective passages, inoculated onto insect and avian cell monolayers, and allowed to replicate for 48 hr. Culture supernatants were harvested and the number of virions was quantified by duplicate plaque assays on BHK-21 cells. Differences in virus production between passaged groups were analyzed by a factorial ANOVA (Steele and Torrie 1980).

Patterns of virus growth: Growth patterns were determined by 24-hr growth curves. After the tenth selective passage, three randomly selected lineages from each selection series and three replicates of the ancestral virus stock were inoculated onto insect and avian cell monolayers following the procedures previously outlined for the selective passages. Aliquots of the culture media were taken every 2 hr for 24 hr, and the titer of free virions (*i.e.*, those present in the culture media not in the cells) was determined by duplicate plaque assay on BHK-21 cells. Differences in growth patterns between the treatment groups for each cell type were analyzed using a factorial ANOVA (Steele and Torrie 1980).

Ability to infect insect cells: The techniques of Johnston and Smith (1988) were modified and used to test the ability of the derived lineages to infect the C7-10 cell line. Approximately 200 virions were inoculated onto C7-10 insect cell monolayers (MOI < 0.0001) and given 1 hr at 34.5° to penetrate the cells. Excess medium was removed, and the cells were exposed to trypsin for 5 min to detach cells from the culture plate and to remove any virions adsorbed to cells. Remaining virions that had not entered cells were removed by two rounds of low-speed centrifugation. Washed cells were resuspended in fresh media and distributed as 75-μl aliquots into multiple 96-well plates containing BHK-21 cell monolayers. The plates were examined at 72 hr for cytopathic effects, and the number of virions that had entered insect cells was determined by the number of wells in which cytopathic effects in BHK-21 cells were observed. Concurrent plaque assays of each inoculum on BHK-21 cell monolayers were used to determine plaque-forming units in the original sample. For each lineage, relative infectivity was calculated as the total number of positive wells divided by the total number of plaque-forming units. In all statistical analyses, assays were blocked by time. Data was rank transformed and analyzed by ANOVA, and the means were compared by LSD (Glantz 1992). Comparable assays to examine the relative abilities of each lineage to infect avian (PDE) cells were also attempted.

RESULTS

Differentiation during serial passage: Cytopathic effects were used to verify viral replication and were detected for every lineage at every passage indicating that no replication failures or lineage extinctions occurred. However, decreases in cytopathic effects were observed in 3 of the 10 insect cell adapted lineages (lineages designated as 1, 2, and 3). Results of a factorial analysis of the mean $TCID_{50}$ values in BHK-21 cells that were observed for each of the three passage groups over the course of the 10 selective passages (raw data not shown) are summarized in Table 1. Although the growth temperature, MOI, and replication period were equal for each of the 30 lineages, significant differences in growth patterns were detected indicating that virus divergence had occurred during 10 selective passages. Virus lineages adapted to the type of cell(s) through which they were passaged ($P = 0.001$). Adaptation was time dependent in that not all lineage groups adapted equally over time ($P < 0.0001$), and adaptation was interactive with cell type ($P < 0.0001$).

Relative detection of viruses in different cell types: Overall differences in the abilities of the insect, avian, and mammalian cell types to detect virions are shown

TABLE 1

Factorial analysis of variance of $TCID_{50}$ values observed for 30 EEE virus populations over the course of 10 serial passages

Source of variation	*F* value	Probability
Type of cell(s)	9.05	0.001
Adaptation over time	56.70	<0.0001
Interactions between cell type and time	10.04	<0.0001

Analysis was performed using the $TCID_{50}$ values in BHK-21 cells that were observed for each of the 30 derived lineages over the course of 10 selective passages through insect cells or vertebrate cells or in alternation between both cell types.

in Table 2. Highly significant differences were detected between cell types in their abilities to detect viruses generated by each of the three selective regimes. Initial side-by-side titrations ($n = 8$) in which the ancestral virus stock was used as a control population indicated that significant differences between cell types existed even in the absence of any host-specific viral adaptation, in that the insect, avian, and mammalian cell lines each registered a different level of virus within identical samples of the population ($F = 99.11$; d.f. 2, 21; $P < 0.0001$). The lowest estimates of control virus were produced by the PDE avian cell line, C7-10 insect cells registered the next highest mean value, and BHK-21 mammalian cell line was most susceptible to infection and replication. When similar assays were performed with derived lineages that had undergone selective passages, significant differences in cell susceptibility were again observed (insect adapted: $F = 4.75$; d.f. 2, 27; $P = 0.0171$; avian adapted: $F = 55.17$; d.f. 2, 27; $P < 0.0001$; alternating: $F = 23.08$; d.f. 2, 27; $P < 0.0001$). As with the control population, the highest mean levels of virus replication were detected by the BHK-21 cell line and the lowest by the PDE line. Because the BHK-21 cell line consistently detected the highest mean number of virions, these cells were used for all further quantitative assays.

Plaque morphology and size: Significant changes in plaque morphology occurred during the 10 selective passages (Table 3). The ancestral control population formed uniformly large smooth plaques on BHK-21 cells. After 10 passages, the plaques of one insect, two avian, and three alternated lineages were indistinguishable from those of the ancestral control. However, the remaining derived lineages displayed a variety of plaque types. The minimum number of distinct plaques within a lineage was one for populations that were completely homogeneous. The maximum number observed within a single lineage was three. On average, virus lineages that were adapted to insect cells had the most diversity in plaque types per lineage and those adapted to avian cells the least.

When the mean plaque size of each tenth passage population was compared to that of the ancestral control, significant decreases in size were detected in 100% (10/10) of the insect-adapted, 80% (8/10) of the avian-adapted, and 60% (6/10) of the alternated lineages. Differences in mean plaque size on BHK-21 cells were also detected between the three treatment groups (ANOVA; $F = 6.68$; d.f. 2, 27; $P = 0.004$). Lineages adapted to growth in insect cells had the greatest average reductions in plaque size.

TABLE 3

Mean number of plaque morphologies and relative plaque sizes of tenth passage EEE virus lineages from each selective regime

Passage history	Mean no. of plaque types	Mean plaque size
Avian cells	1.4^{b} (0.16)	79.34^{a} (4.47)
Insect cells	2.1^{a} (0.28)	9.60^{b} (5.81)
Alternated cells	1.5^{ab} (0.17)	84.66^{a} (4.96)

Mean plaque size was measured relative to that of the ancestral control population and is expressed as a percentage of that value. Values within a column that do not share a common letter are significantly different at the 0.05 level by the LSD method. Means are shown as (±SEM).

TABLE 2

Relative abilities of insect (C7-10), avian (PDE), and mammalian (BHK-21) cell lines to detect virions

	Assay cell type		
Passage series	C7-10	PDE	BHK-21
Insect cells	7.77^{a} (0.60)	7.05^{b} (0.37)	7.89^{a} (0.57)
Avian cells	7.79^{b} (0.16)	7.49^{b} (0.10)	8.89^{a} (0.06)
Alternating	8.49^{a} (0.11)	7.32^{b} (0.17)	8.66^{a} (0.14)
Ancestral control	8.87^{b} (0.09)	8.23^{c} (0.03)	9.46^{a} (0.06)

Lineages were sampled after 10 selective passages through insect cells or vertebrate cells or in alternation between both cell types. Values shown are mean $TCID_{50}$ (±SEM) calculated for 10 lineages in each experimental group and eight replicates of the ancestral control population. Mean values within a row that do not share a common letter are significantly different from each other by the LSD procedure at the 0.05 level. Statistical comparisons are valid only between cell types (rows) and cannot be made between treatment groups (columns).

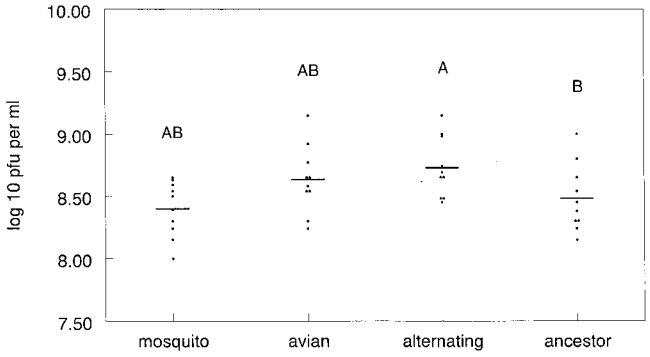

FIGURE 1.—Forty-eight-hour EEE virus production on the Peking duck embryo cell line at 34.5°. Experimental lineages were adapted to growth on insect cells or vertebrate cells or in alternation between these two cell types for 10 selective passages. Values are shown for each of the 10 lineages in a treatment group. Bold horizontal lines represent the mean of the 10 replicates. The means of groups that do not share a common letter are significantly different at the $P < 0.05$ level.

Similar passage history did not result in complete phenotypic convergence. A high degree of within treatment variation in plaque size was confirmed by Kruskal-Wallis analysis for all three passage groups, indicating unequal divergence of lineages maintained under common selective pressures (insect adapted: $H = 104.005$, d.f. 9, $P < 0.0001$; avian adapted: $H = 96.129$, d.f. 9, $P < 0.0001$; alternating: $H = 115.229$, d.f. 9, $P < 0.0001$).

Virus yield from different cell types: When tested on the PDE avian cell line, significant differences in virion production were detected among treatment groups (Figure 1). Lineages that had alternated between cell types produced significantly more virions than did the ancestral stock on PDE cells. Significant differences were also detected when similar assays were performed using the C7-10 insect cell line (Figure 2). Lineages

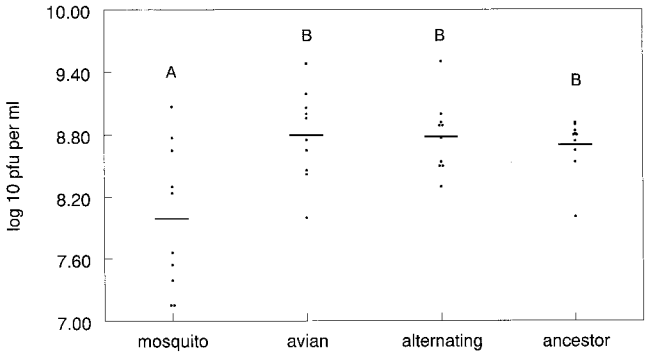

FIGURE 2.—Forty-eight-hour EEE virus production on the C7-10 mosquito cell line at 34.5°. Experimental lineages were adapted to growth on insect cells or vertebrate cells or in alternation between these two cell types for 10 selective passages. Values are shown for each of the 10 lineages in a treatment group. Bold horizontal lines represent the mean of 10 replicate lineages. The means of groups that do not share a common letter are significantly different at the $P < 0.05$ level.

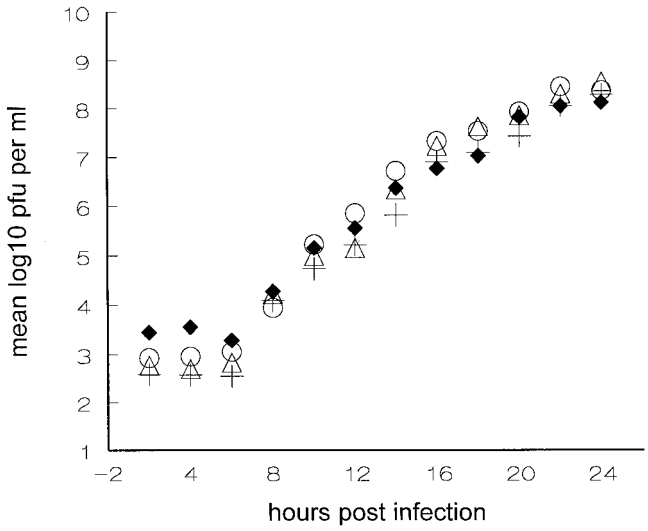

FIGURE 3.—Twenty-four-hour EEE virus growth curves on the Peking duck embryo avian cell line at 34.5°. After 10 selective passages, three randomly chosen lineages were tested from each of the three treatment groups, along with three replicates of the ancestral virus population. Data shown for each time point are mean values calculated from the three chosen lineages. (+) Mosquito, (△) avian, (○) alternating, (◆) ancestor.

adapted to growth on insect cells had >10-fold reduction in virus yield from C7-10 cells compared to lineages from the other three passage series. No significant reductions in virus yield from insect cells were observed for lineages adapted to avian cells or alternating between avian and mosquito cells. Virus populations that alternated between insect and avian cells grew well in both cell types.

Temporal growth patterns on different cell types: The 24-hr growth curves of three randomly chosen virus lineages from each of the passage series on both avian and insect cells are summarized in Figures 3 and 4, respectively. An overall analysis of variance for both sets of growth curve data revealed that the 10 selective passages had resulted in significantly altered temporal growth patterns (Table 4). These results confirmed that lineages adapted to the different cell culture regimes grew differently and that these differences were dependent on passage history and the type of cells used in the growth curve assay. Significant time by treatment and time by cell type effects ($P = 0.03$ and 0.0017, respectively) further confirmed the existence of different temporal growth patterns.

The most striking differences in growth patterns were observed during replication on insect cells (Figure 4). During the eclipse phase (0–4 hr postinfection) insect-adapted lineages appeared highly infectious to the insect cells. Virion production by insect-adapted lineages on insect cells began to level off at ~16 hr postinfection whereas those of the other treatment groups continued to climb before leveling off at ~22 hr postinfection.

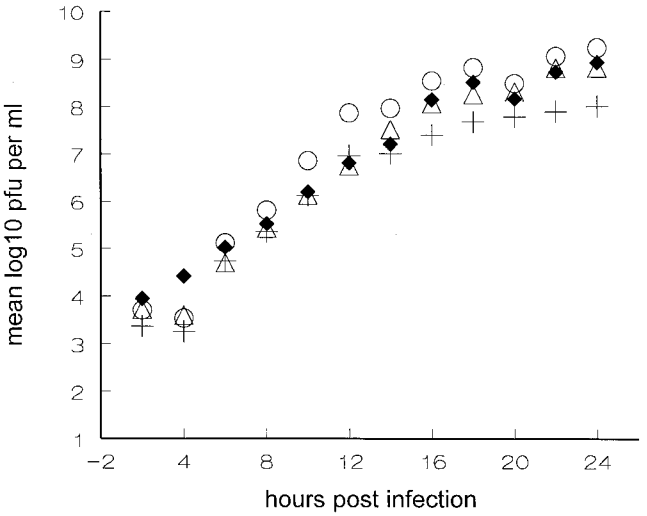

FIGURE 4.—Twenty-four-hour EEE virus growth curves on the C7-10 mosquito cell line at 34.5° ($n = 3$). After 10 selective passages, three randomly chosen lineages were tested from each of the three treatment groups, along with three replicates of the ancestral virus population. Data shown for each time point are the mean values calculated from the three chosen lineages. (+) Mosquito, (△) avian, (○) alternating, (◆) ancestor.

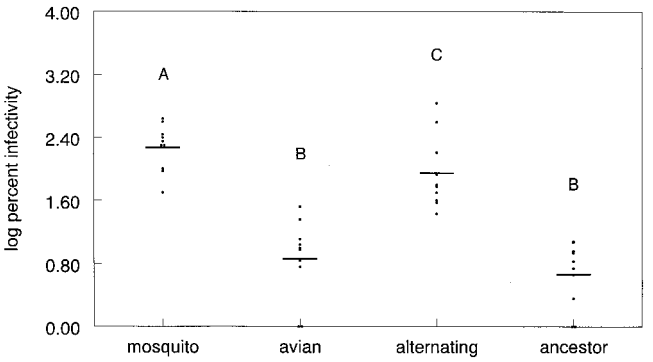

FIGURE 5.—Relative abilities of derived EEE virus populations (tenth passage) to infect C7-10 mosquito cells at 34.5°. Log transformed data are shown for each of the 10 lineages in a treatment group. Values ≤1% are shown as 10^0. Bold horizontal lines represent the mean of the 10 replicate lineages. The means of groups that do not share a common letter are significantly different at the $P < 0.05$ level.

This resulted in a difference in virion production after 24 hr, at which time the number of virions produced by the insect-adapted lineages was an order of magnitude lower than those of the other treatment groups. An analysis of all pairwise comparisons revealed that the growth patterns of the insect-adapted lineages were significantly different from those of all other treatment groups. Significant differences were also detected between the ancestral control population and all three experimental groups when assayed on insect cells.

Ability to infect insect cells: Significant differences (Block $F = 2.85$, d.f. 9, $P = 0.0168$; Treatment $F = 61.22$, d.f. 3, $P < 0.0001$) were observed when derived and control virus populations were tested for their ability to initiate an infection in C7-10 insect cells (Figure 5). When compared to their ancestral control, lineages adapted to replication on insect cells were, on average, almost 40 times as infectious to insect cells. Likewise, virus lineages that had replicated on insect cells at every other passage exhibited dramatic gains, averaging an 18-fold increase in infectivity. Interestingly, for both of these treatment groups the number of infectious particles that were detected in the insect cell assay was often above the independent estimate of BHK-21 plaque-forming units used to measure initial virus input. This gave rise to percentages of >100% infectivity, indicating that more virions were able to infect insect cells than were detected by the BHK-21 plaque assay system. In contrast, the 10 lineages passed only through avian cells did not evolve to be highly infectious to insect cells and had mean infectivity scores that were not statistically different from that of the ancestral control population ($P = 0.13$).

To determine if their increased ability to infect insect cells was due to immediate prior growth in this cell line, three randomly chosen insect-adapted lineages (lineages 5, 7, and 10) were back-passed once through the PDE avian cell line and then retested against the control population. All three lineages continued to display significant increases in insect cell infectivity following a single passage through avian cells (Wilcoxon signed-rank test; $Z = -2.195$; $P = 0.0282$), thus supporting the hypothesis that genetic adaptation(s) to infect insect cells had occurred during the 10 selective passages.

Comparable assays to examine the relative abilities of each lineage to infect avian (PDE) cells were attempted. However, our infectivity assay is dependent upon isolating a single infected cell. The highly adhesive properties of the avian cells made it impossible for us to carry out this assay because we were not able to achieve adequate separation of the monolayer into distinct single cell units.

TABLE 4

Overall factorial analysis of variance for viral growth patterns observed during the first 24 hr of replication in avian (PDE) and insect (C7-10) cells

Source of variation	d.f.	*F* value	Probability
All sources	15	192.52	0.0001
Passage history of lineage	3	3.77	0.0112
Growth curve cell type (avian or insect)	1	92.28	<0.0001
Time of assay (2–24 hr)	1	2613.69	<0.0001
Interactions between time and passage history	3	3.02	0.0304

Virus populations were sampled every 2 hr during the assay. Only significant main effects and interactions are shown.

DISCUSSION

We found that EEE virus populations are capable of rapid evolution in response to new cellular environments, that different selective pressures exist for virus replication in vertebrate and invertebrate cells, and that transfer in a two-host cycle selects for virus populations that are well adapted for replication in both hosts. Results from our study add detail to those previously reported by other investigators (Novella *et al.* 1995, 1999; Weaver *et al.* 1999) in confirming that virus evolution was not random. Overall, our results indicate that virus populations from the same selective regime were more similar to each other than they were to those with different passage histories. Common growth traits in lineages from the same selective regime suggest that each group of 10 lineages may have followed a fairly consistent pattern of differentiation in response to common selective pressures. Such similarities most likely arose in response to selection for virions with increased replicative fitness (Bull *et al.* 1997).

Interestingly, despite their common ancestry and subsequent exposure to identical passage conditions, considerable within-treatment variation occurred among the 10 derived lineages in each selective regime. Heterogeneity among similarly passaged virus populations may reflect nonsynchronous adaptation and be the result of premature sampling of continually evolving virus populations. In other words, 10 selective passages may not have allowed each lineage enough time to maximize its fitness in a new cellular environment. If this is true, further passages may result in more consistent patterns of adaptation and perhaps more homogeneous lineages (Newman *et al.* 1985; Novella *et al.* 1995; Elena *et al.* 1996). Alternatively, additional passages could result in the emergence of phenotypically and/or genotypically distinct virus lineages, each of which followed a different evolutionary course to optimize its replicative fitness. Under this scenario, the heterogeneity within a treatment that we detected after only 10 selective passages may reflect the evolution of lineages that utilized multiple solutions to adapt to their common cellular environment. In either case, additional studies that examine the growth characteristics and the genetic makeup of these evolving virus lineages over time are needed to resolve these issues.

Because our initial study design included a relatively large number of replicate lineages ($n = 10$) for each cell type, we were able to measure within treatment variation and gain insights into the processes of arbovirus host-associated adaptation. In the future, we can study specific virus lineages, such as those that lie at the extreme ends of the phenotypic spectrum. A drawback of our study is that some of the virological assay systems we used were scored on a log scale. Given this, we predict that the small but consistent differences we observed in virion production and growth pattern will be correlated to rather large fitness differences when a more sensitive assay system is employed (Holland *et al.* 1991). We did, however, measure fecundity and infectivity separately, both of which can contribute to overall viral fitness. The advantage of such an approach for an arbovirus system is that by examining the biological properties of evolving virus populations, we gained insights into traits that can influence the pathogenesis or host range of this medically important group of viruses (Crill *et al.* 2000).

Our results indicate that there are at least two mechanistic components of EEE virus evolution: high fecundity and rapid colonization. Increased virion production may be advantageous in two ways. First, if multiple virions simultaneously infect a naive host, their progeny may directly compete for the remaining cellular resources. Virions that are able to overwhelm their competitors by quickly achieving numerical superiority will have an increased chance of colonizing the remaining uninfected cells (Olmsted *et al.* 1984). Second, large virus populations within infected hosts may enhance transmission to the next host in the transmission cycle (Chamberlain and Sudia 1961). Mosquito infection is known to be dose dependent (Hardy *et al.* 1983; Hardy 1988), and the induction of high viremias in infected avian hosts is expected to favor transmission to mosquitoes (Strauss and Strauss 1994). High virus production in mosquitoes will similarly be beneficial for transmission. Rapid expansion of EEE virus populations in mosquitoes increases the likelihood that virions will successfully infect vector salivary glands and be transmitted to a susceptible vertebrate host (Weaver *et al.* 1993a; Scott *et al.* 1994; Strauss and Strauss 1994). Thus, a high rate of virion production is advantageous in both arthropod and vertebrate hosts. Interestingly, we observed that alternated lineages had the highest average virion production on both cell lines.

Increased insect cell infectivity appears to be an important EEE virus adaptation. We found that insect-adapted and alternated lineages increased their efficiency of infection for insect cells. Conversely, the ancestral virus and lineages adapted to growth only in avian cells remained relatively noninfectious. Viruses adapted to growth in insect cells may be more infectious because they are better able to locate and attach to the limited number of available receptors (Ludwig *et al.* 1996). It is unfortunate that we were technically unable to run similar assays in our chosen avian cell line to determine if similar adaptations occurred in lineages adapted to these cells.

Selection among EEE virus populations for rapid entry into host cells and rapid virus replication is consistent with biological evidence from whole animal studies that competition among virions is an important process in arbovirus evolution (Scott *et al.* 1994). The course of alphavirus amplification in vertebrate hosts is often explosive and predominantly limited by the onset of an immune response. Infection of mosquito vectors is life-

long, but the ability to transmit virus declines over time (Scott *et al.* 1994). Superinfection inhibition among closely related viruses is a common phenomenon in cell culture systems and can occur within infected mosquito vectors (Stollar and Shenk 1973; Peleg and Stollar 1974; Davey *et al.* 1979; Karpf *et al.* 1997; L. A. Cooper and T. W. Scott, unpublished data). For this reason primary colonization of certain cell types may be an advantage for transmission. For example, rapid colonization of mosquito salivary gland cells presumably prevents superinfection by competing virus genotypes and is highly adaptive because it accelerates virus transmission.

The observed decreases in total virus production within the insect-adapted lineages when grown in insect cells may have been favored by a negative association between total virion production and the speed of colonization. This kind of negative association is supported by temporal patterns of EEE virus infection and growth in C7-10 mosquito cells (Figure 4). In insect cells, where receptors are relatively rare (Ludwig *et al.* 1996), selection appears to favor rapid entry into cells over the ability to grow to high titer.

The low level of virus production in insect cells by viruses adapted to that cell type was unexpected. Our experimental design ruled out that low yields were attributable to complicating factors such as host range mutants, defective interfering particles, or immediate past replication in insect cells. Furthermore, our results are not unique to EEE virus. Similar decreases in viral yield following insect cell adaptation have been reported for Sindbis virus (Hertz and Huang 1995). Likewise, attenuated, rapidly penetrating Sindbis virus variants also display cell-specific changes in infectivity (Baric *et al.* 1981).

Adaptation of viral populations to multiplicity of infection and the presence of competing virion populations has been well documented in a variety of other RNA virus systems (Sevilla *et al.* 1998; Turner and Chao 1998; Turner *et al.* 1999). Strong intrahost competition between virions can explain the apparently nonadaptive decreases in fecundity observed for insect cell adapted lineages. We speculate that viruses that have incurred a fitness cost in fecundity have gained a fitness advantage through superior colonization ability, a situation that may or may not result in a net loss of virus fitness. The exact relationship between these two traits and their effect on overall virus fitness deserves additional study. Interestingly, this hypothesized trade-off was not seen in lineages that were alternated between insect and avian cells. Alternating EEE virus lineages increased insect cell infectivity while maintaining a high rate of replication on both cell types.

Although multiple lines of evidence suggested that differential evolution occurred between the insect- and avian-adapted EEE virus lineages, we found no evidence that replication in one host system negatively affected performance in the other. In this regard, our results agree with those of Novella *et al.* (1999) but are different from those of Weaver *et al.* (1999). Both of these recently published studies used an experimental design similar to ours to examine host-specific evolution in populations of vesicular stomatitis virus (VSV) and EEE virus, respectively. What differ among the three studies are the number of replicate lineages, the number of selective passages, and the methodology used to assess viral fitness.

Despite their differences, all three studies found that alternated virus lineages performed well in both vertebrate and invertebrate cells. By using standard virological methods, we found that viruses adapted to alternation between cell types had high virion production on both cell types and also gained in their ability to infect insect cells. Novella *et al.* (1999) similarly reported that alternated populations of VSV displayed increased fitness on both vertebrate and invertebrate cell types and concluded that slow rates of arbovirus evolution are not necessarily due to an adaptive compromise for virus replication in different cell types. Weaver *et al.* (1999) also reported that alternating replication in dual hosts increased virus fitness in either host alone.

For alphaviruses, dual hosts, most often mosquitoes and vertebrates, appear necessary for sustained virus amplification (Scott *et al.* 1994). Because they are restricted to obligate cycling within a two-host transmission cycle and they have the ability to rapidly adapt to new cellular environments, it is not surprising that alphaviruses have evolved to maximize their fitness in a two-host life cycle.

Although we found that different selective pressures exist for viruses that are restricted to replication in only one host, lineages grown in alternation between hosts expressed intermediate phenotypes consistent with dual adaptation to both cellular environments. We conclude that different selective pressures exist for alphavirus replication within each component of their two-host transmission cycle and that alternation between hosts selects for virus populations that are well adapted for both.

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