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PRINCIPAL INVESTIGATOR: James L. Hardy
Edward J. Houk

CONTRACTING ORGANIZATION: University of California
Berkeley, California 94720

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VECTOR COMPETENCE OF MOSQUITOES FOR ARBOVIRUSES

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James L. Hardy
Edward J. Houk

University of California
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Infection rates of Aedes aegyptus to dengue 2 viral infection following feeding on artificial bloodmeals were improved by modifying the composition of the artificial bloodmeals. Analysis of these results was improved by reducing the days of extrinsic incubation from 11 to 9, thus avoiding the significant mortality (often 75%) which occurred between days 9 and 11 and minimizing variability from experiment to experiment. A monoclonal antibody has been isolated and used to purify WS2 and WR2 antigens, which are specific to virus susceptible and virus refractory strains of Culex tarsalis, respectively. It was suggested that the nascent WR2 antigen is processed to the WS2 form through posttranslational modification. Hybridomas from mice immunized with WR2 antigen were produced, however, no hybridoma subclones producing antibody were detected. Clones of either of two cell lines may provide a cell culture model for alphaviral titer modulation. Congenic strains of Culex tarsalis which differ in their ability to modulate alphaviral titers were developed. However, the non-modulating strain is also altered in peroral susceptibility so further selection might be needed to yield the desired genotypes.

Alphavirus; RAI; Entomology; Vector Competence; Gut Barrier;
Genetics; Mosquitoes; Monoclonal antibodies

Unclassified Unclassified Unclassified Unlimited

I. Summary

The peroral infection of *Aedes aegypti* females with dengue 2 (den2) virus presented in an artificial bloodmeal has proven to be extremely difficult. In an attempt to circumvent this problem, an attempt was made to increase the available titer of den2 virus through blind passage adaptation to BHK-21 cells. This, however, proved to be futile. After 8 passages, den2 viral titers has been increased only 10-fold. The increase was not likely transient; titers at each of the passage steps varied randomly between no or a 10-fold increase.

Information from the literature indicated that maximal titers of den2 virus can be observed only after 11 days of extrinsic incubation at 32°C. One very significant problem was that, in our hands, this protracted extrinsic incubation period resulted in approximately 90% mortality in some mosquito strains. We attempted to address this problem by examining the temporal variation in infection rates through a 13 day extrinsic incubation period. The results indicated that the percent infected mosquitoes reached a maximum by 5 days post infection and that additional days of incubation were superfilous.

Specific mesenteronal proteins from both refractory (proteins R1 and R2) and susceptible (combined proteins S1/2) mosquito strains were separated by SDS-PAGE, electroeluted and used as specific immunogens to induce antibody production in mice. A number of potentially significant antibodies were produced as determined by both enzyme immune assay (EIA) and western blotting. Rather striking differences in antibody affinity in either EIA or western blotting indicates a high degree of variation in epitope specificity within the monoclonal antibody library. These differences may prove to be invaluable in future studies of protein-virus interaction.

Cell clones that varied in their ability to support the replication of western equine encephalomyelitis (WEE) virus were developed from the *Aedes albopictus* Singh cell line. The observed variation in WEE viral titers between these cell clones was about 10 million-fold. A number of clones (10) were isolated that yielded WEE viral titers of $\leq 1.0 \log_{10}$ PFU/ml. An equal number of cell clones (13) were isolated that allowed WEE viral titers to reach $\geq 8.0 \log_{10}$ PFU/ml. These results would indicate that within *Ae. albopictus* cells a potential model for modulation of viral titer might be developed.

FOREWORD

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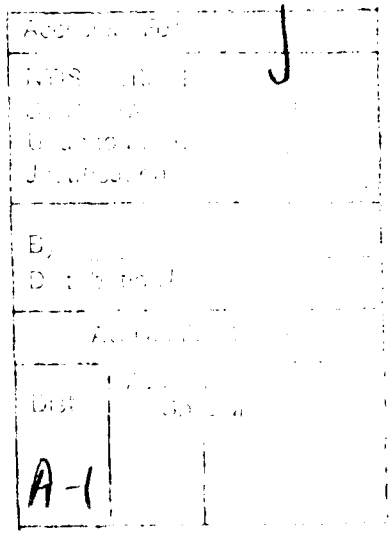

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Research Staff:

James L. Hardy, Ph.D. - Professor of Medical Virology, Principal Investigator
Edward J. Houk, Ph.D. - Associate Research Entomologist, Project Leader
Laura D. Kramer, Ph.D. - Assistant Research Virologist
Robert Bloyer, B.S. - Staff Research Associate I
Betty A. Penny - Laboratory Assistant

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II. Introduction

The susceptibility of an individual mosquito to infection with an arbovirus following feeding on a potentially infectious bloodmeal, and the subsequent transmission of that arbovirus, depends upon a number of intrinsic factors, some of which apparently are under the genetic control of the mosquito. Vector competence is the relative measure of the ability of a mosquito population to become infected with, and to subsequently transmit an arbovirus. Thus, vector competence is the sum of the effects of these intrinsic factors on individual mosquitoes within a population for a particular arbovirus.

A number of years ago, the sequence of events that occurs in a mosquito during arboviral infection was broken down into a number of critical points, where intervention by the host might result in failure to complete the viral infection cycle (Hardy et al., 1983). These sites of potential disruption of the viral infection within the mosquito were termed barriers to infection. The barriers were designated: mesenteronal infection barrier (MIB), mesenteronal escape barrier (MEB), salivary gland infection barrier (SGIB) and salivary gland escape barrier (SGEB). The main focus of this research program has been to identify mosquitoes that exhibit a breakdown in the viral infection cycle and to determine at which point (barrier) the breakdown occurs.

Only interspecific mosquito models were available to elucidate the mechanisms for various barriers to arboviral infection, in the early years of vector competence studies (Houk et al., 1986; Hardy, 1990). In recent years, these initial models have been largely supplanted by more genetically relevant intraspecific models (Kramer et al., 1989; Hardy, 1990). Studies in our laboratories have resulted in the identification of potential intraspecific mosquito, *Culex tarsalis*, model systems for the MIB, MEB and SGIB to western equine encephalomyelitis (WEE) viral infection or transmission. Genetic studies have resulted in the establishment of two well defined intraspecific models for the MIB and MEB to WEE virus.

During the period of this contract, research endeavors have concentrated on the development of models of viral infection barriers in the mosquito, *Aedes aegypti*, to the flavivirus, dengue 2 (den2). In addition, distinct biochemical differences in the proteins found in the brush borders of MIB model *Cx. tarsalis* have been exploited to produce (poly)monoclonal antibodies in an attempt to determine the potential involvement of these proteins in the attachment/penetration of WEE virus into mesenteronal epithelial cells. Finally, one aspect of the MEB in *Cx. tarsalis* has been been at the forefront of study; the potential involvement of a host factor that interferes with (i.e., controls) the replication of WEE virus. A host factor with similar properties has been

identified in some lines of *Aedes albopictus* cell cultures. The *Ae. albopictus* (Singh) cell line has been subcloned by limiting dilution to produce modulating and nonmodulating cell lines.

III. Mosquito Colonies Maintained for Vector Competence Studies

A number of mosquito species and strains that are relevant to our studies on the vector competence of mosquitoes for both alpha- and flaviviruses are maintained in our insectary. In addition, a number of these strains represent unique genetic strains of *Cx. tarsalis* that are exemplary of barriers associated with the inability to become infected with and the subsequent failure to transmit WEE virus.

During the past three years, ten geographic strains of *Ae. aegypti* have been examined for their susceptibility to peroral infection with den2 virus and, because of their extreme refractoriness, have been deleted. A final decision was not made with regard to the strain to be used for selection of the MIB model. The decision was narrowed to a choice between the Davis and Rockefeller strains, both strains were very similar in their peroral susceptibility to den2 viral infection.

TABLE 1. Mosquito colonies maintained for vector competence studies.

SPECIES	COLONY DESIGNATION
Aedes aegypti	Davis, Rexville, Rockefeller, Bangkok, Jakarta, Vero Beach, Miami
Aedes dorsalis	Ft. Baker
Culex peus	Grasshopper Slough
Culex pipiens	Poldervaart
Culex quinquefasciatus	Kern
Culex tarsalis	Chico; Ft. Collins; Knights Landing; Poso Creek· Manitoba; Yuma; WR-1 (FC-KL); WR-2 (FC-KL-C); WS-2 (KL); WS-3; LVP; L-HVP; HVP (KL); HVP (PC); HVP (Y)

IV. Vector Competence Studies of *Aedes aegypti* and Dengue 2 Virus

A. Developmental studies

1. *Blind passage of dengue 2 virus in BHK-21 cells* - A comparison of the relative infectiousness of bloodmeals composed of den2 virus derived from various sources indicated that the percent of mosquitoes infected following feeding on den2 viral suspensions from BHK-21 cells was almost as high as the percent infected following feeding on den2 viral suspensions from C6/36 *Ae. albopictus* cells (Ann. Prog. Rpt., 1989). This observation might not seem so startling but these two viral preparations differed by more than 3 $\log_{10}$ PFU/ml, when assayed in Vero cells. An attempt was made to adapt den2 virus to BHK-21 cells by a series of eight blind passages. The relative titers of den2 virus in the resultant cell culture supernatants was determined by plaque assay in vero cells. Dengue 2 virus failed to adapt to BHK-21 cells following this procedure. The den2 viral titer in the first passage was essentially equivalent to that from the eighth passage (Table 2).

TABLE 2. Response of dengue 2 (New Guinea C) viral titers to blind passage in BHK-21 cells

Passage Number	Viral titer*
1	2.0
2	3.1
3	3.3
4	3.1
5	2.0
6	3.1
7	2.0
8	3.1

2. *Temporal analysis of dengue 2 viral infection of Aedes aegypti* - Watts et al. (1987) reported that in order to allow den2 infections to reach their maximum level of detectability in *Ae. aegypti*, the mosquitoes should be incubated for 11 days at 32°C following peroral infection. This incubation regimen has been followed in a number of our experiments. However, after 11 days at 32°C, mortality rates in excess of 90% are not uncommon with all of the strains of *Ae. aegypti*

used in our experiments. This mortality most often occurred during the period from 9-11 days extrinsic incubation, with the greatest mortality occurring on day 10. These two experiments were undertaken to determine the earliest day that infection with den2 virus could be detected in the Rockefeller strain of *Ae. aegypti* and to determine if den2 viral infection rates could be determined prior to the period of mortality.

The results were quite interesting and somewhat contradictory to the reports in the literature (Fig. 1). Den2 viral infection was detected as early as day 3. Contamination by den2 virus retained within the lumen of the digestive tract might present a problem in determining primary infection of the mosquito at this early time point. However, there was no visual evidence of remnants of the bloodmeal in the mosquito's digestive tract and the possible contribution of den2 virus as a contaminant was regarded as minimal. Primary infection of mosquitoes was obvious as the percent infected increased from 15% to 35% between days 3-5. In fact, on day 5 postinfection, the percent infection was the higher than any other day during the experimental period. The percent

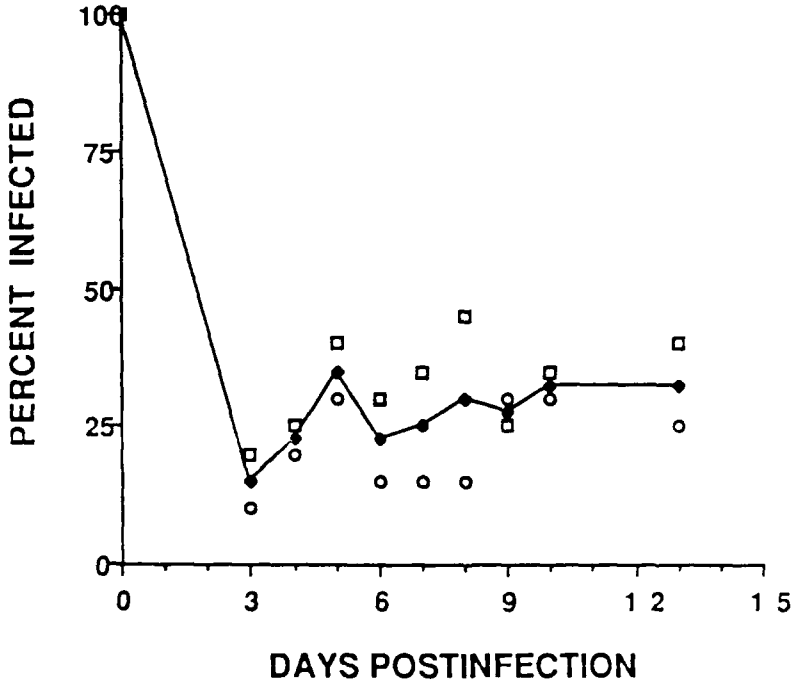


Figure 1. Temporal variation in the percent infected *Aedes aegypti* following peroral infection with dengue 2 virus (New Guinea C) prepared from C6/36 *Aedes albopictus* cell cultures (Miller et al., 1982).

infected fell slightly during the next few days and leveled off between days 8-13 at approximately 30.0%. There was a large variation between the two experiments in the percent infected during the period of 6-8 days but this was reduced to a stable level of percent infected on days 9 and 10.

It would appear that the determination of the percent infected *Ae. aegypti* could be reliably determined as early as 5 days following peroral infection with den2 virus (Fig. 1). If one is concerned about residual bloodmeal (i.e., viral) contamination, the percent infected could be very reliably and reproducibly determined 8 or 9 days following peroral infection. One thing is very obvious from these two experiments: the percent infected can be reliably determined several days before significant mortality occurs.

B. Comparison of three strains of *Aedes aegypti* (Davis, Rexville and Rockefeller) following ingestion of a den2 viral bloodmeal

Artificial bloodmeals to be fed upon by the mosquito, *Ae. aegypti*, were prepared according to Miller et al. (1982), with some modification in an attempt to increase den2 viral titers. Cells (BHK-21 or C6/36) were infected at a multiplicity of infection of 1.0 and incubated for 6 days at 28°C for C6/36 and 4 days at 37°C for BHK-21 cells. Cells were scraped from the surface of the culture vessels and divided into 2 equal aliquots. Each aliquot was centrifuged at 1500xg-10 min to pellet the cells and the supernatant was carefully aspirated. The cell pellet of one aliquot of each of the cell types was resuspended in 0.1x volume of the original, clarified supernatant. The remainder of the supernatant was concentrated by centrifugation in a filter that resulted in a greater than 10-fold concentration of the supernatant (Ann. Prog. Rpt., 1988; 1989). This concentrated supernatant, 0.1x the original volume, was used to resuspend the second aliquot of cells. Infected cells resuspended in 0.1x volume clarified supernatant [CS], filter concentrated infectious supernatant [C] and infected cells resuspended in 0.1x volume of concentrated infectious supernatant [CS/C] provided the three different feeding suspensions for each of the two cell types. The artificial bloodmeals were prepared by mixing den2 viral suspensions (0.1 ml), human red blood cells freshly prepared by twice washing in phosphate buffered saline (0.4 ml), fetal bovine serum (0.45 ml) and sucrose (60% w/v; 0.05 ml). The mixtures were placed as droplets directly on the mesh of the feeding containers.

Prior to feeding on an artificial bloodmeal mosquitoes were deprived of sucrose for 48 hr and H_2O for 24 hr. Mosquitoes were given a 30 min during which to engorge from the potentially infectious bloodmeals. Engorged mosquitoes were identified and separated from unengorged

mosquitoes with the aid of a microscope, following anesthetization with CO_2. Mosquitoes were held at 32°C for 11 days (Watts, et al., 1987) and then assayed for den2 viral infection by plaque assay in Vero cells (Ann. Prog. Rpt., 1988).

1. *Engorgement rates* - A comparison of engorgement rates was made based upon the cell type in which den2 virus was grown, the type of feeding suspension fed upon and the geographic strain of *Ae. aegypti* (Table 3). There was no evidence that any of the three strains of *Ae. aegypti* preferentially fed to engorgement on any one of the six different artificial bloodmeals prepared from either BHK-21 or C6/36 cells. Concentrated supernatant prepared from infected cells (C) yielded a significantly higher percentage of engorged females when compared to den2 infected cells suspended in unconcentrated supernatant (CS; $t_{0.05}$=2.80>2.15) and to den2 infected cells resuspended in concentrated supernatant (CS/C; $t_{0.05}$=2.95>2.15). There was a significant difference in the engorgement rates between the three geographic strains of *Ae. aegypti*. The Rexville strain was the best feeder; significantly higher engorgement rates than the Davis strain ($t_{0.01}$=5.95>>2.98) and the Rockefeller ($t_{0.05}$=2.14) strains. Further, the engorgement rate for the Davis

TABLE 3. Comparison of the engorgement rates for three laboratory strains of *Aedes aegypti*, allowed to feed to engorgement on dengue 2 viral infectious bloodmeals of various compositions prepared from BHK-21 and C6/36 cells (Miller et al., 1982).

Feeding suspension*	Mean percent mosquitoes feeding (Total number tested)** Rexville	Rockefeller	Davis
BHK-21			
[C/CS]	65.3 (150)	68.7 (160)	39.3 (150)
[CS]	76.3 (150)	70.7 (160)	48.0 (150)
[C]	80.3 (150)	78.0 (160)	68.0 (150)
C6/36			
[C/CS]	70.5 (100)	53.0 (110)	36.0 (100)
[CS]	68.5 (100)	43.0 (110)	48.0 (100)
[C]	85.0 (100)	66.0 (10)	48.0 (100)

* [C]=Feeding suspension prepared from filter concentrated, infectious cell culture supernatant.
[CS]=Feeding suspension prepared by the resuspension of infected cell pellet in 0.1x of the original volume of infectious cell culture supernatant.
[C/CS]=Feeding suspension prepared by the resuspension of infected cell pellet in 0.1x of the original volume of filter concentrated infectious cell culture supernatant.

** The data were combined from three separate feeding experiments. Viral titers ingested between the three different experiments varied by less than 0.5 $\log_{10}$ PFU/ml.

strain was significantly higher than that observed for the Rockefeller strain ($t_{0.01}$=3.72>2.98). This latter observation is somewhat interesting, since the Davis strain was derived from the Rockefeller strain (Dr. Bruce Eldridge, University of California, Davis).

2. *Mortality rates* - Mortality rates for *Ae. aegypti* females infected with den2 virus have historically been extremely high during the extrinsic incubation period of 11 days at 32°C (Ann. Prog. Rpt. 1988; 1989). This particular duration and temperature of extrinsic incubation were chosen based on published data that analyzed this combination of factors to allow optimal detection of den2 viral infection rates in *Ae. aegypti* (Watts et al., 1987). The described experiments examined the effects of mosquito strain, bloodmeal composition and cell culture origin of den2 virus on mosquito mortality (Table 4). Essentially, there were no statistical differences in mosquito mortality between the two (i.e., BHK-21 and C6/36) cell culture sources for den2 virus or between the three (i.e., C, CS and C/CS) different bloodmeal compositions. An apparently greater mortality was observed in the Davis strain compared to the Rexville strain but this was not

TABLE 4. Comparison of the mortality rates for three laboratory strains of *Aedes aegypti* allowed to feed to engorgement on dengue2 viral infectious bloodmeals of various compositions prepared from BHK-21 and C6/36 cells (Miller et al., 1982) and incubated for 11 days at 32°C.

Feeding suspension*	Mean percent mosquito mortality (Total number tested)** Rexville	Rockefeller	Davis
BHK-21			
[C/CS]	47.3 (106)	33.9 (100)	71.3 (58)
[CS]	33.4 (123)	42.3 (106)	41.5 (72)
[C]	24.2 (130)	46.3 (108)	53.6 (104)
C6/36			
[C/CS]	6.8 (79)	51.0 (61)	59.2 (38)
[CS]	23.2 (72)	25.2 (43)	39.1 (58)
[C]	43.3 (94)	45.9 (67)	22.9 (26)

* [C]=Feeding suspension prepared from filter concentrated, infectious cell culture supernatant.
[CS]=Feeding suspension prepared by the resuspension of infected cell pellet in 0.1x of the original volume of infectious cell culture supernatant.
[C/CS]=Feeding suspension prepared by the resuspension of infected cell pellet in 0.1x of the original volume of filter concentrated infectious cell culture supernatant.

** The data were combined from three separate feeding experiments. Viral titers ingested between the three different experiments varied by less than 0.5 $\log_{10}$ PFU/ml.

statistically significant at the 5% level. This was the only mosquito strain related difference in mortality observed in these experiments.

3. *Peroral infection rates* - The data from three different experiments were combined to determine if bloodmeal composition, cell culture source for den2 virus and/or mosquito strain differences could be detected for infection rate (Table 5). Infection rates were significantly lower when the den2 virus was grown in BHK-21 cells, compared to den2 virus grown in C6/36 cells ($t_{0.01}$=3.70>2.90). This could easily be related to viral titer since the titer for feeding solutions prepared from BHK-21 cell derived den2 virus typically was more than 2.0 $\log_{10}$ PFU/ml lower than that determined for den2 virus grown in C6/36 cells (Ann. Prog. Rpt., 1989). In fact, although the experiments have not been completed, it would appear that percent infection/PFU might be greater for BHK-21 derived den2 virus than for C6/36 derived virus (Table 5; Ann. Prog. Rpt., 1989).

TABLE 5. Comparison of the peroral infection rates for three laboratory strains of *Aedes aegypti* allowed to feed to engorgement on dengue2 viral infectious bloodmeals of various compositions prepared from BHK-21 and C6/36 cells (Miller et al., 1982) and incubated for 11 days at 32°C.

Feeding suspension*	Mean percent mosquito infection (Total number tested)** Rexville	Rockefeller	Davis
BHK-21			
[C/CS]	5.6 (30)	7.4 (51)	46.5 (9)
[CS]	10.7 (56)	7.5 (53)	15.5 (31)
[C]	4.9 (61)	9.9 (43)	22.6 (41)
C6/36			
[C/CS]	16.6 (72)	13.7 (24)	62.5 (15)
[CS]	30.9 (53)	30.0 (32)	30.6 (35)
[C]	6.6 (48)	11.6 (34)	33.0 (20)

* [C]=Feeding suspension prepared from filter concentrated, infectious cell culture supernatant.
[CS]=Feeding suspension prepared by the resuspension of infected cell pellet in 0.1x of the original volume of infectious cell culture supernatant.
[C/CS]=Feeding suspension prepared by the resuspension of infected cell pellet in 0.1x of the original volume of filter concentrated infectious cell culture supernatant.

** The data were combined from three separate feeding experiments. Viral titers ingested between the three different experiments varied by less than 0.5 $\log_{10}$ PFU/ml.

A highly significant difference in peroral infection rates based on mosquito strain was observed (Table 5). The Davis strain of *Ae. aegypti* had a peroral infection rate that was much higher than either the Rexville ($t_{0.01}$=4.27>>3.11) or Rockefeller ($t_{0.01}$=3.83>3.11) strains. There was no significant difference in the peroral infection rates of the Rexville and Rockefeller strains. A ranking of peroral susceptibility to den2 infection would be Davis>Rockefeller>Rexville. This sequence might have been anticipated since the Davis strain has been derived from the Rockefeller strain.

Examination of the peroral infection rates of mosquitoes based on bloodmeal composition revealed the following relationship: C/CS>CS>C (Table 5). However, the only statistically significant difference was between C/CS and C ($t_{0.05}$=2.21≥2.20). There were obvious differences between CS and the other two bloodmeal compositons but these were well within the bounds of random differences.

These feeding, mortality and infection rate studies revealed a number of interesting relationships between cell culture origin of den2 virus, composition of the infecting bloodmeal and strains of *Ae. aegypti* (Tables 3, 4, 5). The Rexville strain fed most readily of the three mosquito strains tested but it was not very susceptible to peroral infection. The bloodmeal composed of filter concentrated cell culture supernatant (C) was more readily fed upon than bloodmeal composed of pelleted infected cells (C/CS and CS). However, the C bloodmeal was the least infectious of the three compositions. There were no differences associated with the cell culture origin of infectious virus until one compared infection rates; virus derived from C6/36 cells was significantly more infectious than that derived from BHK-21 cells. However, as pointed out in the above discussion, this difference is most likely related to the much lower titer of den2 virus found in BHK-21 cells, as compared to C6/36 cells. Based on these studies, the Davis strain fed on a C/CS bloodmeal derived from C6/36 cells would provide one with the greatest number of infected mosquitoes following an 11 day extrinsic incubation period at 32°C.

V. Studies on the Vector Competence of *Culex tarsalis* for Western Equine Encephalomyelitis Virus

A. Mesenteronal infection barrier

Differences in the peroral susceptibility of mosquito strains/species to infection by arboviruses have been well documented (Hardy et al., 1983; Hardy, 1990). In those vector-virus systems initially examined, differences in peroral susceptibility have been shown to be genetic and

to reside within the microvillar membrane of mesenteronal epithelial cells. In our own studies, we have been able to investigate both inter- and intraspecific models for the peroral susceptibility of *Cx. tarsalis* to WEE virus (Houk et al., 1986; Kramer et al., 1989). These model systems, along with the development of a method through which the microvillar membranes could be isolated from dissected mosquito mesenterons (Houk et al., 1986), have allowed biochemical comparisons of the protein composition of these membranes to be attempted.

The comparison between the electropherograms of the proteins of microvillar membranes from susceptible and refractory mosquitoes yielded two predominant proteins that piqued our interest. One protein of approximately 100kD is found at the same cross-reference point in 2D electropherograms in both the susceptible and refractory components of our inter- and intraspecific MIB models (Ann. Prog. Rpt., 1988; 1989). However, a second protein varies in a consistent manner between the susceptible and refractory components of our MIB model systems. In microvillar membranes from refractory mosquitoes, a protein of approximately 150 kD is observed, while in susceptible mosquitoes a second protein of approximately 100 kD is observed (Ann. Prog. Rpt., 1989).

We have attempted to charactaerize differences between microvillar membranes from susceptible and refractory mosquitoes by immunological methods (Ann. Prog. Rpt., 1988; 1989). It has been our contention that a clearer understanding of the relationships between the variable proteins in susceptible and refractory mosquitoes might be derived from these types of studies. Our initial immunological studies used crude preparations of microvillar membranes as the immunogen (Ann. Prog. Rpt., 1987). In recent years, we have become more specific in our choice of immunogens. Specific proteins are electroeluted from electrophoretic gels, washed twice with PBS by centrifugal, molecular size filtration and subsequently used as immunogen (Ann. Prog. Rpt., 1988; 1989).

1. *Monoclonal antibodies to brush border fragment proteins* - During this contract period, we have characterized one of the antibodies derived from immunization with microvillar membrane fragments. This particular antibody (13A5.8) has been well characterized and shown to be a very specific antibody with application in affinity column isolations of mesenteronal antigens from both susceptible (S2 antigen) and refractory (R2 antigen) mosquito mesenterons (Ann. Prog. Rpt., 1989). This particular antibody has been produced in gram quantities by the Hybridoma Laboratory (Dr. Alex Karu) as part of an evaluation of bioreactor production of monoclonal antibodies (Ann. Prog. Rpt., 1989).

2. *New monoclonal antibodies* - We have tried several different times during this contract period to produce monoclonal antibodies against specific proteins from refractory and susceptible mosquito microvillar membranes. Initially, we used mascerated acrylamide gel fragments containing the protein of interest as the immunogen. This procedure elicited a poor antibody response in the inoculated mice. As a consequence, fusion efficiency in the production of antibody producing hybridomas was essentially nil. We obtained only one antibody producing hybridoma (32G8), from two different fusion attempts. However, this hybridoma has proven to be extremely weak and has not been successfully subcloned, to date.

During the final year of this contract, we have concentrated our efforts on the use of specific protein immunogens separated by SDS-PAGE electrophoresis and subsequently electroeluted from the acrylamide gels. The immunogens were designated as R1, R2 and S1/S2; the letter designation indicates the mosquito strain of origin (R=refractory; S=susceptible) and the numerical designation suggests the hypothetical relationship between proteins derived from the two mosquito strains. This hypothetical relationship has been extensively discussed (Ann. Prog. Rpt., 1989). To quickly review, SDS-PAGE electrophoresis allows one to separate the two proteins of interest found in the microvillar membranes of refractory *Cx. tarsalis* because of their large difference in molecular size: the 100 kD protein was designated as R1 and the 150 kD protein as R2. The presumed homologous proteins from the microvillar membranes of susceptible *Cx. tarsalis* cannot be separated by SDS-PAGE electrophoresis, since both are approximately 100 kD. As a consequence, the immunogen preparation from susceptible mosquitoes was a combination of the two proteins, S1/S2. It was anticipated that by these having three separate immunogen preparations we would be able to derive a sufficient number of uniquely reacting and cross-reactive monoclonal antibodies so that the relationship between these two pairs of presumed protein relatives from susceptible and refractory mesenterons could be demonstrated.

Two different strains of mice, B10.Q and Swiss/Webster (S/W), were immunized with purified antigens. The B10.Q mouse was a mouse that had previously been immunized with mascerated acrylamide gel fragments (Ann. Prog. Rpt., 1989) but not involved in a fusion to produce hybridomas. The S/W mice (2) were newly immunized with only the electrophoretic antigen diluted in PBS and Ribi adjuvant The effectiveness of the immunization protocol was determined by enzyme immune assay (EIA) of tail bleed sera from these mice (Fig. 2).

Our EIA screens indicated that the R1 and S1/S2 antigens were not effective in eliciting a good immunological response in the mice (Fig. 2A, 2C). On the otherhand, the R2 antigen elicited a very good response in S/W mice and a lesser response in the B10.Q mouse (Fig. 2B). Since the

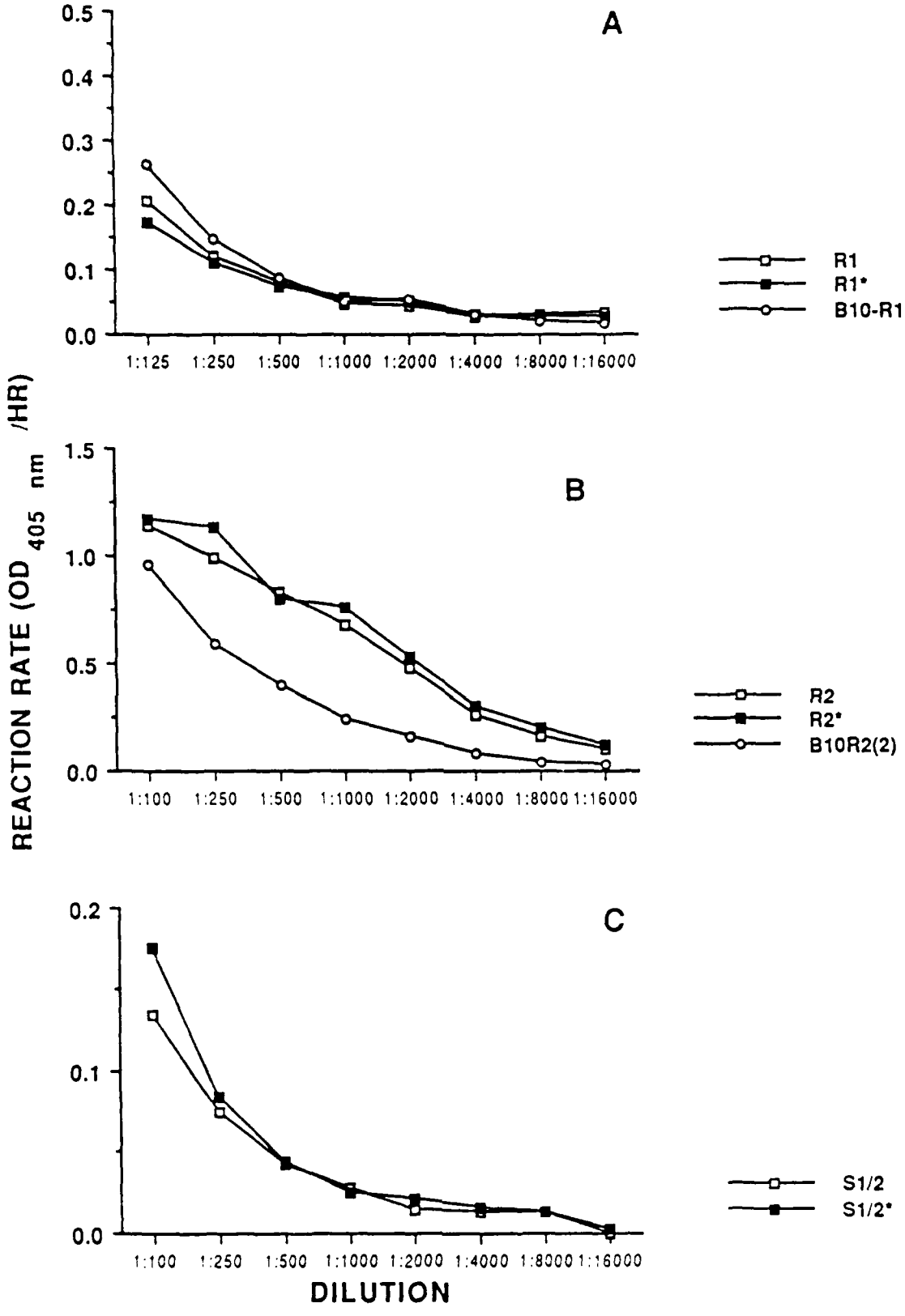
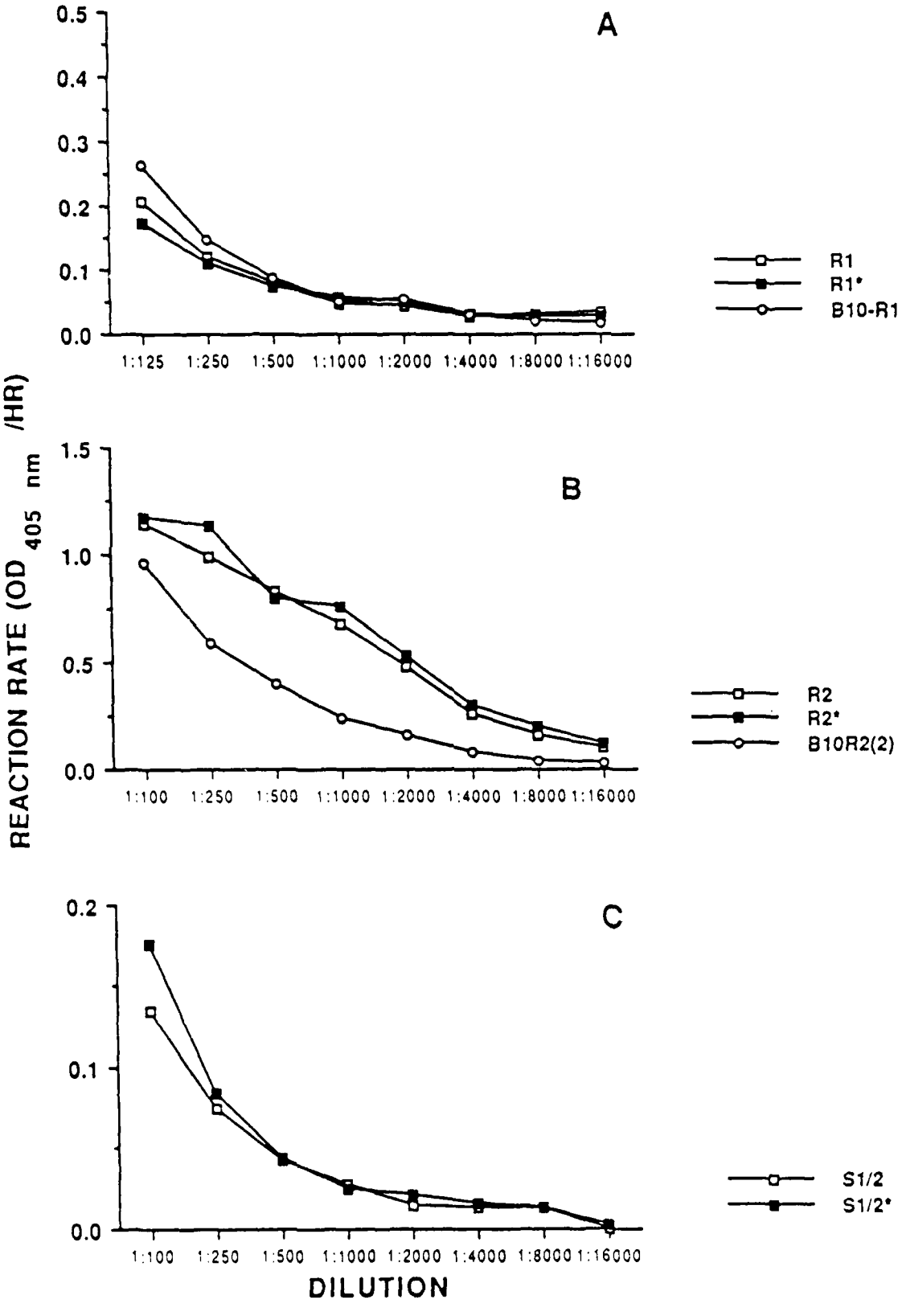


Figure 2. The serum antibody responses of the various mice inoculated with electroeluted antigens (A) R1 antigen, (B) R2 antigen and (C) the combined S1/2 antigens. All mice were Swiss/Webster except for those designated as B10.Q.



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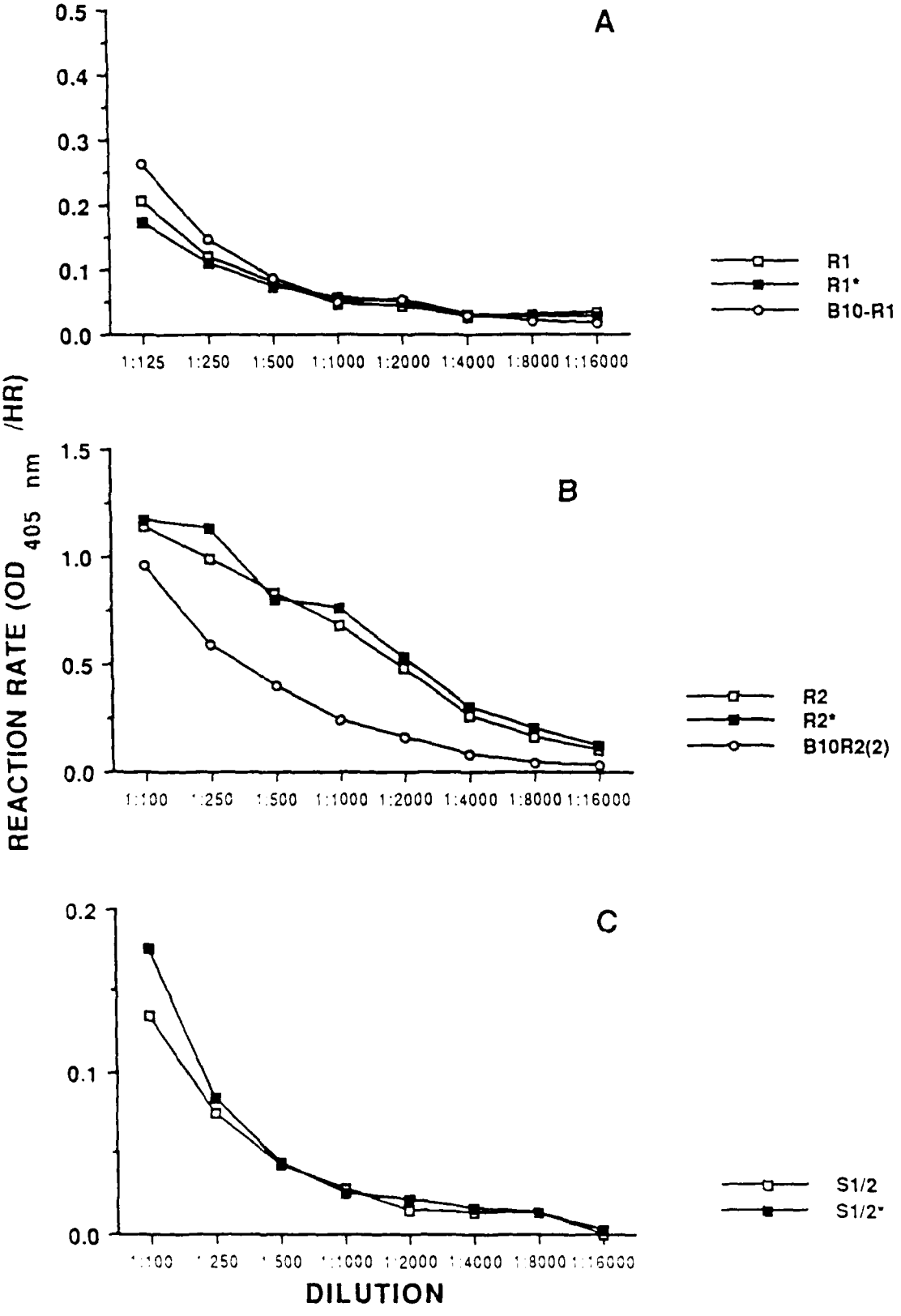
A
0.5
0.4
0.3
0.2
0.1
0.0
1:125 1:250 1:500 1:1000 1:2000 1:4000 1:8000 1:16000
R1
R1*
B10-R1
B
1.5
1.0
0.5
0.0
1:100 1:250 1:500 1:1000 1:2000 1:4000 1:8000 1:16000
R2
R2*
B10R2(2)
REACTION RATE (OD 405 nm /HR)
C
0.2
0.1
0.0
1:100 1:250 1:500 1:1000 1:2000 1:4000 1:8000 1:16000
S1/2
S1/2*
DILUTION

B10.Q was getting quite old and it could not be used to produce ascites antibody, this mouse and one of the S/W were chosen to be included in the first fusion using electroeluted antigen.

The results of this fusion were excellent. Almost 3000 hybridomas were obtained and screened from the two mice. This remarkable number of hybridomas was partially attributed to a change in the protocol for producing hybridomas in the laboratory. In the place of polyethylene glycol fusions between spleen cells and myeloma cells, electrofusion was used. The number of myelomas required is significantly reduced and the fusion efficiency is greatly enhanced (Dr. Alex Karu, Pers. Commun.), as is evident from the large number of hybridomas screened. The hybridomas were first screened by EIA in order to eliminate those hybridomas secreting nonspecific antibodies. Subsequently, those hybridomas secreting specific antibodies as determined by EIA were further screened by western blot (Tables 6A; 6B).

Four of the most promising hybridomas from the S/W fusion were subcloned. These hybridomas showed a significant reduction in EIA activity following expansion into 24-well tissue culture plates and were immediately subcloned in an attempt to rescue them. Of the four, only 52C1 was successfully rescued.

Isotyping determinations were made on the hybridoma supernatants from 24-well expansion plates. In a number of cases, the reaction rate at this point was significantly reduced, indicating that these particular hybridomas had either ceased to produce antibodies or were being overgrown by nonproducing hybridomas. In either case, there was an immediate need for subcloning in an attempt to rescue antibody secreting hybridoma clones. Those hybridomas of greatest importance are generally those in the IgG subclasses. IgM subclass antibodies are of lesser importance because of their restricted utility, especially in the realm of affinity columns for antigen isolation. Six excellent IgG_1 producing hybridomas were identified among the S/W hybridomas, all of these had reaction rates in excess of 0.800 abs/hr. Three IgG_{2a} producers were identified but their rates were somewhat reduced; the three best were between 0.600 -0.750 abs/hr; one of these was from the B10.Q fusion (14C3). In the IgG_{2b} subclass, five candidate hybridomas were identified but only three of the five had rates in excess of 0.500 abs/hr. Only two IgG_3 subclass producing hybridomas were identified as possible candidates for subcloning. Three candidate IgM subclass producing hybridomas were identified and 67F4 had an especially high EIA reaction rate (≈1.800 abs/hr).

TABLE 6A. Compilation of the antibody producing hybridomas obtained from a B10.Q mouse immunized with R2 antigen and a summary of their 96-well activities.

Hybridoma designation	Reaction Rate	Reaction rate to background ratio	Western blot	Antibody isotype
2A11	3.20	999.9	0	$IgG_{1a,2b,3}$
8A7	1.92	274.6	+++	
8H7	13.47	999.9	++	
11E4	3.00	999.9	+++	
11E10	1.04	20.1	0	IgG_{2a}
14C3	4.13	28.9		IgG_{2a}
17B7	2.14	999.9		IgG_{2a}
17H6	4.62	999.9	0	IgM
20G3	3.24	999.9	+	
22E12	3.28	999.9	+/-	
23A1	6.60	999.9	0	IgG_{2a}*
24C8	2.53	26.7	0	
26B10	2.15	999.9		
27H3	10.74	34.5		
28C4	2.82	999.9		
28C8	11.90	36.9		
30F8	2.49	999.9		
34H6	2.21	999.9		
36B7	2.12	28.3		
37B12	4.70	24.4		
37C4	2.14	999.9		
37D10	4.56	82.8		

*Diminishingly low reaction rate during antibody subtyping.

Several antibody producing hybridomas were observed that yielded excellent antigen detection through western blots but had very low, or undetectable, EIA rates (Tables 6A; 6B). Some of these will be subcloned in an attempt to determine if their western blot activity might be enhanced. It is interesting to note at the western blot activity (epitopes) appears to be distinct from the EIA activity (epitopes). This relationship could be further explored in an attempt to use these antibodies to examine the relationships between mesenteronal antigens in *Cx. tarsalis*.

B. Modulation of WEE viral titers in mosquito cell cultures

1. *Cloning of Aedes albopictus (Singh) cells-Aedes albopictus* cells were grown to confluence in L-15 medium in a T-25 flask. The cells were freed from the substrate by repeated pipetting, viable cell counts determined with a hemocytometer and the cells diluted to a density of 1 cell/0.2 ml in conditioned medium. Aliquots of 0.2 ml were distributed into each well of approximately 20 96-well tissue culture plates in an attempt to maximize the probability of monoclonality (Tooker and Kennedy, 1981).

TABLE 6B. Compilation of the antibody producing hybridomas obtained from a Swiss/Webster mouse immunized with R2 antigen and a summary of their 96-well activities.

Hybridoma designation	Reaction Rate	Reaction rate to background ratio	Western blot	Antibody isotype
41E3	4.99	20.5		
42A1	2.17	999.9	0	IgG2b
42B8	3.85	33.1	+/-	IgG1**
45F11	4.24	999.9	0	
46A2	2.43	29.6		
46G5	3.23	999.9	+/-	
48B12	10.80	234.8		
48D10	2.02	72.1		IgG3
48F10	2.33	999.9	+	IgM
48F11	2.17	20.5		
48H6	2.75	44.3		
49B7	1.67	26.4	+/-	
49G7	18.40	36.4		IgG1
50C9	3.10	999.9	+/-	
50E8	1.22	999.9	+++	
50F3	1.21	999.9	++	
51G11	3.45	39.2	0	
52C1	9.10	999.9	+	IgG1**
53G9	8.11	999.9		IgG2b
57A12	10.40	21.7		IgM
58F1	21.43	15.1		
59B9	3.75	24.3		
60F8	4.72	999.9		
60G1	15.43	60.3		IgG1
62F1	1.70	154.5		IgG2b
62F4	3.98	361.5	++	
63A1	4.55	66.9		
63F12	1.89	999.9	0	IgG2a**
64A11	8.72	999.9		
64F12	2.01	34.1	+++	IgG2b**
64G3	7.12	37.5		IgM(?)*
64G5	1.24	999.9	+++	
66D8	6.08	999.9		IgG2b
66D12	6.56	999.9		
66F12	3.05	999.9	+	
67B11	7.76	999.9		IgG3
67D12	2.49	999.9		IgG1
67F4	4.54	22.2		IgM

* Diminishingly low reaction rate during antibody isotyping.
** Subcloned.

Cell growth was observed after approximately one week in a number of wells but attrition was significant over the next 2-3 weeks. After a period of 4 weeks, 51 clones were successfully transferred to 12-well plates. Confluent wells were split 1:3 with two plates used as duplicates for freeze-down in liquid N_2 and the third to test WEE viral growth.

Cloned cells were infected with WEE virus at 1.0 MOI. After 72 hr, cell sulture supernatants were harvested and viral titers determined by plaque assay in Vero cells. Ten clones

were found to have WEE viral titers of ≤ 1.0 $\log_{10}$ PFU/ml and 13 clones revealed titers of ≥ 8.0 $\log_{10}$ PFU/ml (Fig. 3).

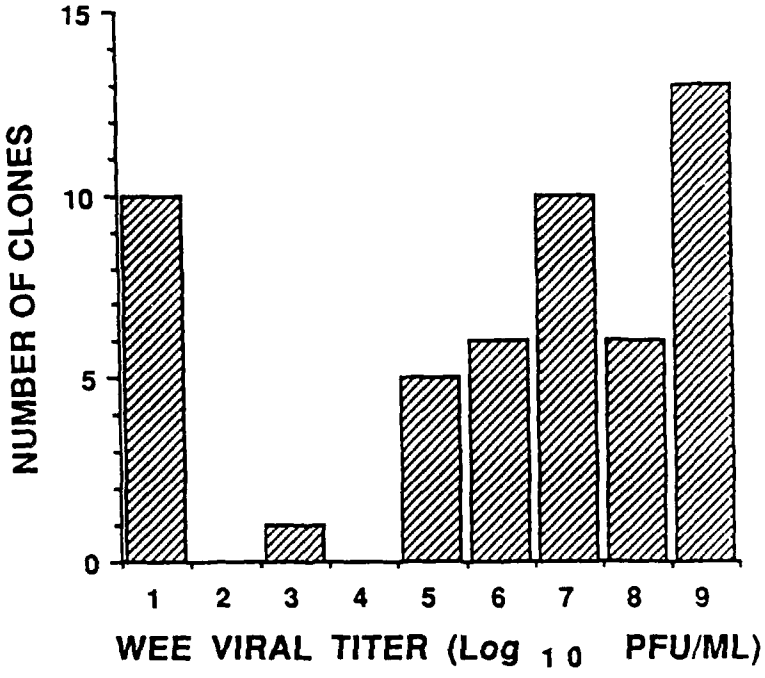

Figure 3. Distribution of western equine encephalomyelitis viral titers in cell clones of *Aedes albopictus* Singh cells.

The results indicate that *Ae. albopictus* cells are a very heterogeneous population of cells that vary significantly in their ability to support the replication of WEE virus. The difference in WEE viral titers between the low producing cell clones and the high producing cell clones as approximately 10 million-fold. This difference is reminescent of the differences noted between low virus producing and high virus producing strains of the mosquito, *Cx. tarsalis* (Ann. Prog. Rpts., 1988, 1989). It would appear that we may be able to develop a cell culture model for viral modulation. A number of subsequent studies will reveal whether this hypothesis can be justified.

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