(2)

DOCUMENTATION PAGE DTIC FILE COPY Form Approved OMB No. 0704-0188

AD-A224 755

DTIC ELECTE AUG 0 3 1990 S B D CS

1b. RESTRICTIVE MARKINGS

3. DISTRIBUTION / AVAILABILITY OF REPORT
approved for public release; distribution unlimited

2b. DECLASSIFICATION / DOWNGRADING SCHEDULE
N/A

4. PERFORMING ORGANIZATION REPORT NUMBER(S)
N/A

5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION	6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
University of California		

6c. ADDRESS (City, State, and ZIP Code)
Berkeley, CA 94720

7b. ADDRESS (City, State, and ZIP Code)

8a. NAME OF FUNDING / SPONSORING ORGANIZATION	8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
U.S. Army Medical Res. & Dev. Command		DAMD 17-87-C-7118

8c. ADDRESS (City, State, and ZIP Code)
Fort Detrick, Frederick, MD 21701-5012

10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
66102A	3MI-61102BS13	AA	057

11. TITLE (Include Security Classification)
Virulence Markers of Dengue Viruses (U)

12. PERSONAL AUTHOR(S)
James L. Hardy, Ph.D. and Srisakul C. Kliks, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual/Final Report	FROM 5/18/87 TO 8/18/89	1990/2/20	48

16. SUPPLEMENTARY NOTATION
N/A

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	03		Dengue viruses, dengue hemorrhagic fever, virulence, virulence marker, monocytes. RA1
06	13		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Illnesses in humans caused by the four serotypes of dengue virus vary from mild forms i.e. pyrexia of unknown origin (PUO) and dengue fever (DF) to severe forms i.e. dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS). One of the factors that contribute to the DHF and DSS disease outcomes is proposed to be the variation in the virulence of dengue viruses. We initially evaluated monocyte-infectivity as a marker the for virulence of dengue-2 virus by testing 72 dengue-2 viral isolates associated with PUO/DF and DHF/DSS obtained from eight geographic locations for their ability to infect and multiply in freshly isolated human monocytes. Dengue-2 viral isolates from DHF/DSS patients exhibited a higher monocyte-infectivity than that of the isolates from PUO or DF patients. Statistical analyses of the data indicated a correlation between the probability of a dengue-2 viral isolate causing DHF/DSS and the viral infection in human monocytes in the presence of dengue enhancing antibodies. Dengue-2
(continued reverse)

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION
unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mary Frances Bostian	(301) 663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 Previous editions are obsolete. SECURITY CLASSIFICATION OF THIS PAGE

AD_____

VIRULENCE MARKERS OF DENGUE VIRUSES

ANNUAL/FINAL REPORT

James L. Hardy and Srisakul C. Kliks

FEB. 20, 1990

Supported by

U.S. ARMY M[illegible]ICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Det[illegible]ick, Frederick, Maryland 21701-5012

Contract No. DAMD17-87-C-7118

University of California
Berkeley, California 94720

I. Statement of the problem

Illnesses in humans caused by the four serotypes of dengue virus include pyrexia of unknown origin (PUO), classical dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The expression of these disease outcomes follows a discernable pattern in regions of the world where dengue viruses are endemic. Variations in the virulence of dengue viruses, as defined by their ability to cause severe illness, is proposed to be one of the factors that contribute to the DHF and DSS disease outcomes. The manifestation of these severe outcomes is also related to host factors. For effective prevention and control of dengue diseases, it is important to identify virulent strains of dengue viruses and to elucidate mechanisms which result in multiple disease forms and patterns. The overall object of this research project was to establish whether monocyte-infectivity of the virus could be used as a marker for dengue virulence. Subsequently, other biological markers which are unique to virulent viral strains and that can be mapped on viral glycoprotein by monoclonal antibodies, will be determined in order to establish the role, if any, of the viral glycoprotein in conferring virulence to the virus. Knowledge of how the viral glycoprotein is involved in the expression of virulence may contribute toward the development of an effective vaccine.

Summary

The objective of the research was to investigate whether monocyte-infectivity can be used as a virulence marker for dengue viruses. For this purpose, virulence is defined as the intrinsic ability of the virus to cause severe forms of dengue illness - dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), rare disease outcomes in contrast to the generally mild febrile illness or classical dengue fever (DF). Since the viral infection in human monocytes has been implicated by numerous epidemiological and experimental observations, as playing an important role in the development of the DHF/DSS, the ability of the virus to infect human monocytes was evaluated for a virulence marker of dengue virus.

The approach was to measure the viral infectivity for and multiplication in human monocytes in the presence and absence of dengue enhancing antibody, and then determine if this correlates with disease outcome (DF or DHF/DSS). Initially, monocyte-infectivity was evaluated as an in vitro virulence marker for dengue-2 virus , then it was further determined if the same marker existed for other dengue serotypes.

Seventy-two dengue-2 viral isolates from various geographic locations were analyzed to determine if there was an association between the clinical disease of patients from which a virus was isolated and the infectivity of the virus for human monocytes. The results indicated that the probability of a viral isolate causing severe illness is correlated with monocyte-infectivity in the presence of enhancing antibodies. Further analysis on the break down of the disease severity into dengue fever and DHF I, II, III and IV caused by viral strains from Thailand, also showed a statistically significant association with antibody-mediated monocyte-infectivity of the virus. Dengue-2 viral strains associated with DHF/DSS exhibited medium to high monocyte-infectivity indices, while those strains associated with PUO/DF exhibited medium to low indices. It was concluded from these analyses that viral infectivity in human monocytes in the presence of enhancing antibodies is not a completely definitive marker for virulence of dengue-2 virus. However, it may serve as a suitable in vitro correlate for elucidation of a more definitive marker(s).

The genetics of dengue-2 virus is diversed. Eleven genetically distinct groups or topotypes have been identified based on the degree of homology of the viral ologonucleotide fingerprint patterns. These topotypes also segregate geographically. The 72 strains of dengue viral isolates evaluated in this study were found to belong to six different topotypes. To determine if genetic variation and monocyte-infectivity of dengue-2 viral strains correlate in any way, we looked for common patterns of distribution of the virulent strains among various topotypes. Dengue-2 viral strains with high monocyte-infectivity were mostly distributed within topotypes unique to Thailand, The

Philippines, and Indonesia where DHF/DSS is prevalent. The remaining isolates exhibiting high monocyte-infectvity were in a topotype unique to Jamaica/ (late) Puerto Rico where DHF/DSS cases have occurred sporadically since 1983. These findings suggested that viral genetics may play a role in the ability of dengue-2 virus to infect human monocytes.

Dengue viral serotypes -1,-3 and -4 each have different geographic distributions from that of dengue-2 serotype and from one another. Epidemics of DHF/DSS associated with these serotypes are less well documented than those observed with dengue-2 serotype. Also, the clinical features of human disease produced by these viruses are slightly different from those associated with dengue-2 virus. Since it was not known if dengue serotypes 1, 3 and 4 have the same pathogenetic basis for virulence as dengue-2 virus, experiments were done to determine if there was a correlation between the infectivity in monocytes and the clinical symptoms associated with selected viral isolates of each of these three dengue viral serotypes. Tests for infectivity in human monocytes in the presence and absence of specific enhancing antibodies were done on 87 appropriate isolates of dengue serotypes -1 ,-3 and -4 . Subsequent analysis indicated a positive correlation between viral infectivity in the presence of enhancing antibodies and severe illness with dengue-4 virus ($p < 0.05$). In contrast, no correlation was observed with dengue-1 and dengue-3 viruses. These findings suggest that different mechanism may be involved in the pathogenesis of dengue serotypes -1 and -3 versus serotypes -2 and -4.

To develop a standardized and rapid method to measure viral monocyte-infectivity, we screened several human monocyte cell lines for dengue-2 viral susceptibility, using the in situ enzyme linked immunoassay (EIA) to detect infection. Human leukemic K-562 and human promonocytic CZ cell lines were shown to be permissive to dengue-2 viral infection, both in the absence of enhancing antibodies. Further evaluation on the use of these cell lines in place of freshly isolated human monocytes indicated that viral infectivity in human K-562 and CZ cells without enhancing antibodies could be used to evaluate dengue viral virulence. However, attempts to measure the extent of viral infection in these cells by the rapid EIA was unsuccessful due to the random loss of cells from the loosely adherent monolayers.

Further characterization of two pairs of virulent and avirulent strains of dengue-2 virus from two topotypes, Thailand and Puerto Rico, was carried out in order to search for a virion property or component responsible for the virulence. It was observed that the viral binding efficiency between the dengue-2 virulent and avirulent strains to monocytes was not different. The kinetics of viral internalization appears to be similar for both the virulent and avirulent strains. However, differences were detected in the pH required for membrane fusion of the virulent and avirulent viral strains. Virulent dengue-2 strains exhibited membrane fusion activity in a slightly more basic pH range than their avirulent counterparts. The infectivity of

avirulent strains also was more sensitive to inhibition by chloroquine than the virulent strains, suggesting that uncoating of the avirulent virus occurred in a more acidic environment than the virulent strains. This change in fusion pH profile may be associated with a genetic change in the amino acid sequence of the viral E glycoprotein responsible for fusion activity.

To investigate whether the variations in the dengue viral E glycoprotein may correlate with the viral infectivity in monocytes, we evaluated the reactivity of the virulent and avirulent strains by three biological tests that involve the E glycoprotein. Differences in neutralization, hemagglutination and antibody dependent enhancement activities were observed suggesting that some variations in the E glycoprotein of virulent and avirulent strains of dengue-2 virus may exist. Further research to determine whether these changes in the E glycoprotein correlate with monocyte-infectivity may lead to the identification of a molecular marker for virulence of dengue-2 virus.

Foreword

For the protection of human subjects the investigators have adhered to policies of applicable Federal Law 45CFR46.

Table of Contents

List of Tables

List of Figures

Research Staff

Srisakul C. Kliks, PhD, Assistant Research Virologist, Project Leader
Charlotte C. Kent, M.S., Graduate Research Assistant
Ryan L. Alfonso, B.S., Staff Research Associate

II. Background

There are two main hypotheses that have been advanced to explain the occurrence of the disease severity (DHF/DSS) resulting from dengue viral infection. One hypothesis suggests that the main risk factor is the presence of preexisting dengue antibodies, acquired passively from the mother or actively from a prior infection with another serotype of dengue virus. (Halstead et al.,1970; Halstead, 1984). The other hypothesis (Rosen 1977) suggests that genetic variability within dengue viral populations gives rise to viral strains with varying degrees of virulence. Although evidence from epidemiologic and experimental studies have partially supported both of these hypotheses, conclusive evidence to prove either hypothesis to be the sole mechanism for the pathogenesis of DHF/DSS does not exist.

Earlier research confirmed that enhancing antibodies are an important risk factor in the development of DHF/DSS in infants and children in Thailand (Kliks et al., 1988; 1989). A similar finding was reported from the 1980 DHF/DSS outbreak in Cuba (Bravo et al., 1987). However, this type of disease pattern was not observed in other dengue endemic areas where there has been opportunity for sequential infections to occur. Kouri et al. (1987) also suggested that viral virulence was an additional risk factor in the development of DHF/DSS. This was further supported by the observation of the intratypic genetic variation among dengue-2 viral strains (Trent et al., 1983; Repik et al., 1983).

A study on replication of several strains of dengue-2 virus from Thailand showed that virus bound and replicated efficiently in a line of Ae. albopictus mosquito cells . However, these viral strains were unable to infect freshly isolated human monocytes due to poor binding (Kliks et al., 1990). However, viral binding was enhanced by dengue-2 polyclonal antibodies and this resulted in a greatly enhanced monocyte-infection. These results demonstrated that the low binding efficiency of the dengue -2 viral strains for human monocytes could be overcome by enhancing antibodies. These observations, specific to dengue-2 virus in Thailand, suggest that the two alternative hypotheses about the occurrence of DHF/DSS are not mutually exclusive since both viral and host factors could be involved. In light of these findings, investigations were undertaken to determine if virulence was correlated to the ability of the virus to infect monocytes in the presence of the enhancing antibodies. Further characterization of virulent and avirulent viral strains was performed to gain insight into the possible molecular basis of dengue-2 viral virulence. The results of these experiments are presented in this Final Report.

III. Assessment of Monocyte-infectivity as a Virulence Marker for Dengue-2 Virus

A. Selection of viral strains

Dengue-2 virus is probably the most virulent serotype since it is frequently associated with DHF/DSS outbreaks. It was the predominant serotype associated with DHF/DSS in Thailand from the mid 1960's to the early 1980's in spite of the cocirculation of other serotypes (Hoke et al., 1983). It was the only serotype responsible for the large DHF/DSS outbreak in Cuba during the early 1980's (Guzman et al., 1984). Furthermore, this serotype is widely distributed in various geographic areas, including those that are not known to be associated with the occurrence of DHF/DSS. Genetic diversity within the dengue-2 serotype has been confirmed and similar variants or topotypes are confined to certain geographic areas (Trent et al., 1983; D.W. Trent, personal communication). In addition to its diverse nature, both genetically and epidemiologically, dengue-2 virus has been well studied with regard to the viral infectivity in monocytes (Halstead et al., 1984; Morens et al., 1987; Kliks et al., 1988). For these reasons, dengue-2 virus was used as a model in studies on the evaluation of monocyte-infectivity as a virulence marker.

The ideal approach would be to compare monocyte infectivity of dengue-2 viral strains isolated from DHF/DSS cases with those associated with pyrexia of unknown origin (PUO) or classical dengue fever (DF) from the same dengue epidemics. However, viral isolates obtained from DHF/DSS endemic regions tend to be limited to isolates from DHF/DSS cases while those from regions where DHF/DSS is sporadic or absent are mostly associated with PUO or classical DF cases. To create a sufficiently large bank of strains that are associated with a variety of disease outcomes, we obtained strains of dengue-2 virus associated with severe illnesses from regions where DHF/DSS is endemic. Conversely, most of the strains associated with PUO or DF included in our study were obtained from locations where dengue viruses are endemic and DHF/DSS cases are sporadic or absent. Attempts were made to include some of the isolates associated with PUO or DF from DHF/DSS prevalent areas. Likewise, we obtained as many isolates as possible of DHF/DSS cases from the DHF/DSS sporadic locations.

All dengue-2 viral isolates were from humans and, with one exception, had less than five passages in mosquitoes and/or Ae. albopictus C6/36 cells. Information regarding their source of isolation, passage history, clinical symptoms, infection status and oligonucleotide fingerprint patterns was stored in a data bank using the dBaseII program. Viral isolates were coded before being tested for their ability to infect freshly isolated human monocytes in the presence and absence of dengue enhancing antibodies.

B. Monocyte infectivity test

One day old monocytes isolated by elutriation technique (Wahl et al.,1984) in the laboratory of Dr. L.H. Wahl at the National Institutes of Health, Bethesda, MD, were air freighted in wet ice overnight to our laboratory. Cells were counted and viability was determined by the trypan blue exclusion method. Cell aliquots containing $6x10^5$ cells were infected at a multiplicity of infection (MOI) of 0.05 and 0.5 with each viral strains. The infection was performed in the presence and absence of mouse polyclonal antibodies to dengue-2 virus at the optimal enhancing dilution. After a four day incubation period at $36^{o}C$, determinations were made on 1) the quantity of virus produced in the monocyte culture and 2) the proportion of infected monocytes as measured by the indirect immunofluorescent assay (IFA). Variations were noted on the intensity and staining pattern with IFA positive cells. Three main IFA patterns were noted; focal, granular and completely diffuse.

C. Correlation between disease outcome and monocyte infectivity of the virus

The variables included in the correlation analysis are listed (Table 1). The clinical diagnoses in this study were not standardized since the diagnosis was determined at the time of the outbreaks by different attending scientists or physicians. Most of the cases from Thailand and those from other countries diagnosed after 1975 are likely to conform with the WHO guideline criteria for diagnosis of DHF/DSS. Thus, although there may be some inconsistencies in reporting the clinical differentiation between PUO and DF vs DHF/DSS, this is not a systemic bias.

The analysis of 72 dengue-2 viral isolates, using both Spearman and Pearson correlation procedures, suggested that the probability of a viral isolate causing severe illness was associated with 1) virus production in the presence of enhancing antibodies , 2) the enhanced quantity of virus produced and 3) the pattern of IFA staining of the infected monocytes in regard to the distribution and intensity of the stain (1+ to 4+) (Table 2). Further analysis using multiple logistic regression, which controlled for the variability of infection rate due to different donors, indicated that the occurrence of DHF/DSS was also associated with the three monocyte infection parameters mentioned above. The probability of a dengue-2 viral strain causing DHF/DSS in a human increased by 32 %, 20 % and 15 % with a unit increase of the virus yield with enhancing antibodies, the enhanced quantity of virus yield and the IFA staining pattern, respectively (Table 2).

All 72 isolates were ranked according to the strongest correlate for monocyte-infectivity, i.e., virus production in the

presence of enhancing antibodies (Table 3A). The results indicate that 100 % of the isolates were associated with a DHF outcome when the virus yield was 6 $\log_{10}$ or greater, 94 % when the yield was 5 $\log_{10}$ or greater and 68 % when the yield was 4 $\log_{10}$ or greater. There was some discrepancy in the ranking due to the variability in monocyte donors. Thus, an infectvity index was created by setting the virus yield of the reference viral strain from each experiment equal to 100. The viral yield of the tested strains were then expressed in relation to this reference index (Table 3B). This standardization resulted in only minor changes in rank of viral isolates. According to the adjusted ranking order, the isolates associated with DHF/DSS exhibited monocyte infectivity indices ranging from 45 to 135 with a mean of 81.92 and S.D of 20.68, while those isolates associated with PUO and DF exhibited the mean infectivity index of 48.17 with S.D. of 20.35 (Figure 1). The difference in the monocyte-infectivity between the two groups was statistically significant ($p < 0.001$).

It was of further interest to determine whether the degree of disease severity (i.e. PUO, DF and the different grades of DHF/DSS) was related to viral yield in the presence of enhancing antibody from infected monocytes. This type of evaluation was possible with only the isolates from Thailand since the clinical evaluation and the assignment of the grade of dengue severity was performed by one physician (Dr. Suchitra Nimmanitya, of the Bangkok Children Hospital). This analysis included 19 viral isolates from Thailand, a location of high prevalence of DHF/DSS. The results from linear regression analyses of these data (Figure 2) showed a moderate ($p < .01$) association between monocyte-infectivity and the six grades of disease severity.

The above findings suggest that 1) monocyte-infectivity is a suitable in vitro correlate for studies to further identify the virulence marker for dengue-2 virus; 2) virulence is defined strictly by the probability of the virus to cause DHF of any grade; 3) monocyte infectivity is directly associated with infection in the presence of enhancing antibodies.

D. Topotypes of virulent dengue-2 viral strains and their geographic distribution

Dengue-2 viral isolates included in this study varied genetically according to the RNase oligonucleotide fingerprint patterns (D.W. Trent, personal communication). These genetic variants have been organized into 11 topotypes based on the degree of the fingerprint homology (Trent et al., 1988). The distribution of each topotype is confined within a unique geographic locations (Trent et al.,1983; D.W. Trent, personal communication). Data from the present study also suggest that the distribution of virulent strains of dengue-2 virus was restricted to certain geographical location or topotypes (Figure 3). Of 72 viral isolates analyzed, 39 came from DHF or DSS patients while 33 came from PUO or DF patients. The 39 viral isolates associated

with DHF/DSS, which exhibited a high monocyte infectivity index of 81.92 ± 41.32, belonged to topotypes 1, (Thailand), 2 (the Philippines), 3 (Indonesia) and 6 (Jamaica/late Puerto Rico) (Trent, 1988)(Figure 3). In contrast, the 33 isolates associated with PUO and DF were randomly distributed in all topotypes. One isolate from Jamaica/late Puerto Rico topotype exhibited a monocyte infectivity index that was higher than the mean + 2 S.D.(Figure 3). The restriction of strains exhibiting high monocyte-infectivity among the topotypes unique to locations where DHF/DSS is prevalent or sporadic suggests a role of viral genetics in the disease outcome.

It is of interest from an epidemiological view point to note the four anomalous strains from the Jamaica/late Puerto Rico topotype with high monocyte infectivity indices. They are: one isolate from Puerto Rico during the year 1986, associated with DHF; one isolate from Trinidad during 1986, associated with DF; and, two isolates from Jamaica during 1983, of unknown clinical outcome (not included in the analysis). The time periods during which these viruses were isolated coincides with the time in Puerto Rico when sporadic cases of DHF/DSS first emerged. A study involving a larger sample with a larger sample size of isolates from this region may enable detection of a relevant genetic variation within the topotype or antigenic variation relevant to virulence. Such information may help explain the changing disease pattern in the Caribbean region as evidenced by the emergence of DHF cases (Dengue Surveillance Summary No. 51, 1988).

IV. Determination of Monocyte Infectivity as a Virulence Marker for Dengue-1,-3 and -4 Viruses.

Observations and studies, implicating the infection of human monocytes in the presence of the enhancing antibodies as an important risk factor in the pathogenesis of DHF/DSS, were limited to dengue-2 virus (Halstead et al., 1977; Burke et al., 1988; Kliks et al., 1988; 1989). It was assumed that DHF/DSS caused by other serotypes was associated with similar pathogenic mechanisms . However, the disease pattern of dengue-1 and dengue-3 viruses appeared to be different from that of dengue-2 virus. For example, at a given equal rate of transmission to humans, dengue-1 virus in Thailand was less frequently associated with DHF/DSS than dengue-2 (Burke et al.,1988). In addition, the incidence of DHF/DSS attributable by dengue-1 and dengue-3 viruses was not associated with the secondary infections (Burke et 1988; Gubler et al, 1979). Furthermore, the clinical manifestations of DHF/DSS found in Indonesia associated with dengue-3 infections exhibited slightly different clinical features (Eram et al., 1979), since they were more frequently associated with hemorrhage rather than hemoconcentration. These observations suggested that pathogenetic mechanism for other dengue serotypes may differ from that for dengue-2 virus.

To test the above hypothesis, studies were done to examine for a correlation between monocyte infectivity of isolates of dengue serotypes -1, -3 and -4 and the associated clinical symptoms. The same approach as described for dengue-2 was employed. However, only preliminary assessments were performed due to the less extensive collection of the isolates as well as the less frequent disease outbreaks caused by dengue-3 and -4 serotypes. In total 87 isolates of dengue-1 ,-3 and -4 viruses from various geographic origin were tested for their ability to infect freshly isolated human monocytes in the presence and absence of dengue enhancing antibodies. Antibody mediated infection was observed for 69 % of 41 dengue-1 isolates. However, there was no correlation between monocyte infectivity, either with or without enhancing antibodies and the associated clinical manifestation (Table 4). In contrast, dengue-3 virus exhibited a relatively low ability to infect human monocytes. Of the 27 isolates tested, only 8 (30 %) exhibited infectivity in human monocytes under the same experimental condition inspite of the association of 70 % of the isolates with severe dengue illness. Thus, there was no correlation between monocyte infectivity and the severity of illness for dengue-3 isolates (Table 4).

Because of the low frequency of dengue outbreaks associated with dengue-4 virus, only 19 isolates from three geographic locations were included in the analysis. Although the level of infectivity, as determined by the percentage of infected cells and titers of virus produced, was lower than that observed with dengue-2 virus, the monocyte infectivity of dengue-4 virus appeared to correlate with the degree of severity of illness ($p < 0.05$) (Table 4). These preliminary findings suggest that pathogenesis of dengue serotypes -1 and -3 may not be related to monocyte infectivity, whereas it appears to be for dengue serotypes -2 and -4.

V. Search for Suitable Mammalian Monocytic Cell Line for the Monocyte Infectivity Test

In this study variability of monocyte susceptibility to a standard strain of dengue-2 virus was observed among different donors indicating that there was an added extrinsic variable in the present evaluation. Therefore, donor variability was statistically controlled for in our analysis. It would be prudent, however, to circumvent the donor-variability and to increase the rapidity and conveniency of the test. In an attempt to accomplish this, an in situ EIA was first developed to detect the viral infection expressed as cell associated antigens in the 96 well cell culture plates. One day old BHK-21 cells grown in a 96 well plate were infected at varying MOI with dengue-2 viral strain 16681 from Thailand. After a 72 hrs incubation period, cell monolayers were washed and fixed with cold methanol for the detection of cell associated viral antigens, as described by Yong-He and associates (1984). This procedure used mouse polyclonal mouse ascitic fluid as the first antibody, goat anti-

mouse Ig as the second antibody and 3'-3', 5'-5'-Tetramethylbenzidine (TMB) as the substrate for the enzyme, peroxidase. Satisfactory results were obtained when the infection was carried out at an MOI ranging from 0.001 to 0.1.(Figure 4).

Next, several mouse and human monocytic cell lines were screened for their susceptibility for dengue-2 viral infection and their ability to adhere to the plastic matrix. The human myelogenous leukemia cell line K-562 (Lozzio & Lozzio, 1979) was suitable because of its susceptibility to dengue-2 viral infection even in the absence of the enhancing antibodies. To promote cell adherance which is essential for accurate detection of viral antigen by EIA, cells were cultured in specially treated tissue culture plates "Primaria" (Falcon product). Approximately 1×10^6 cells per well were plated into the 96 well "primaria" plates. Cell cultures were infected with several isolates of dengue-2 virus at the MOI of 0.1. After four days of incubation at 37° C, the culture fluids were collected for a plaque assay to determine the quantity of virus produced and the cell monolayers were fixed in cold methanol for 30 minutes before they were tested for the expression of viral antigen by in situ EIA described above. Monocyte infectivity in the micro K-562 cell system was compared to the standard system using freshly isolated human monocytes on 15 dengue-2 viral strains. The correlation analysis (Table 5) indicated that the levels of infection in the K-562 monocytic cultures as measured by the amount of virus produced, correlated with the infectivity indices obtained previously with primary human monocytes. However, levels of infection detected by the EIA method was shown to be less consistent than the infectivity indices, evidently, due to random cell loss from the cell (Table 5). Thus improvement on cell attachment is needed for an effective utilization of the plaque quantitation method by the EIA method.

A newly established human promonocytic CZ cell line was obtained from Dr. Wu-Tse Liu of the National Yang-Ming Medical College, Taiwan, and evaluated for its susceptibility for dengue-2 viral infection and their ability to adhere to cell culture plates. The results shown in Table 6 indicated that although CZ cells produced lower viral yields than did K-562 cells, variation in the viral infection was comparable to that detected by primary human monocytes. In addition, partial correlation with the infectivity indices suggested that CZ cells may adhere to the plastic surface better than do K-562 cells.

In summary, K-562 human myelogenous leukemic cell line and human premonocytic CZ cell line are suitable replacement for primary human monocytes for their ability to distinguish the extent of viral infectivity between the virulent and avirulent strains of dengue virus. The use of these cell lines for the purpose of strain characterization eliminates the problem of biological variation among donors mentioned eariler. These cell lines are easily maintained in modified RPMI medium. The infection assay can be performed in small 96 well plates without enhancing antibodies. However, it is not possible at this time

for the infection to be assessed through the production of cell associated antigen detectable by the rapid EIA assay.

VI. Further Characterization of Monocyte Virulence Marker for Dengue-2 Virus.

A. Viral binding, internalization and uncoating studies.

Early events in the viral multiplication cycle such as attachment, penetration and uncoating, can greatly influence the outcome of the infection. To investigate the underlying mechanism for the variability in infection of monocytes with dengue-2 virus (i.e., the basis for virulence) these early events in the multiplication cycle were evaluated and compared using the virulent and avirulent dengue-2 viral strains. Two topotypic pairs of virulent/avirulent of dengue-2 virus were selected for these studies. One pair included dengue-2 strain D80-293, isolated from a DHF case and dengue-2 strain PUO-263 from a PUO case, both associated with secondary infection, during the same 1980 dengue outbreak in Bangkok, Thailand. Dengue-2 strain D80-293 exhibited high monocyte-infectivity index of 100 compared to the low index of 52 exhibited by the strain PUO-263. The other pair included dengue-2 strain PR-742 isolated from a DHF case and dengue-2 strain PR-160 isolated from a DF case, both from the same 1986 dengue outbreak in Puerto Rico. The strain PR-742 exhibited high monocyte-infectivity index of 115 compared to the low index of 36 exhibited by the strain PR-160.

The above viral strains were grown in C3/36 cell cultures and radiolabeled with H^3-Uridine and S^{35}-Methionine. Purified radiolabeled viruses were then tested for their ability to bind to primary human monocytes. No difference in the binding capacity to monocytes was observed between the Thai virulent and avirulent strains as shown (Figure 5) . In the presence of enhancing antibodies, binding capacities of both viruses increased to a similar extent. Despite the similarity in viral binding capacity to monocytes, the infection levels, were differentiated both in the presence and absence of the enhancing antibodies as indicated by the percent infected cells (Figure 6) and viral yields (Figure 7). Similar results were also obtained with the virulent and avirulent dengue-2 viral strains from Puerto Rico (data not shown).

As a control, binding capacities of the virulent and avirulent strains to Ae. albopictus C6/36 cells were also compared. Both viruses bound to mosquito C6/36 cells more efficiently than they did to monocytes but no difference in binding capacitiy between the viruses was observed (Figure 8). In contrast to that observed with monocytes, viral yields from the virulent and avirulent strains were comparable in C6/36 cell cultures (Figure 9). Similar results relating binding and

infection outcome between the other pair, virulent (PR-742) and avirulent (PR-160) strains from Puerto Rico, was also observed.

In summary, the viral binding data obtained with the virulent/avirulent strains indicates that viral binding is not related to the differential infectivity observed between the virulent and virulent strains of dengue-2 virus.

The rate of viral internalization following the antibody-mediated attachment of the virulent and avirulent strains of dengue-2 virus was measured to determine if it accounted for the differential infectivity observed between the virulent and avirulent strains of dengue-2 virus. Purified radiolabeled virus was mixed with an enhancing concentration of dengue-2 mouse polyclonal antibodies and the mixture was then allowed to interact at 0-4^{o}C for 1.5 hrs with freshly isolated human monocytes for attachment via Fc receptors. The residual virus-antibody mixture was washed off and the monocytes were then incubated at 37^{o}C for various time intervals to allow viral internalization. At the end of each time period, the cells were fixed in 1% paraformaldehyde-PBS solution to stop the internalization process. The residual virus was removed by the treatment of cells with proteinase K, followed by glycine-HCl treatment at pH 2 to elute virus from antibody molecules and virus-antibody complexes from the Fc receptors. Identical samples without the treatment represented both the virus that was internalized and that attached on the cell surface. The results indicated that a large degree of viral elution from the cell surface had occurred during the 37^{o}C incubation period. However, a relative rate of viral internalization could be assessed by calculating the ratio of cell associated radioactivity between treated and untreated cells. The results shown in Figure 10 indicated that there was no apparent difference in the relative rate of viral internalization between the virulent and avirulent strains of dengue-2 virus. These preliminary findings suggested that the kinetics of viral internalization via antibody and Fc receptors also may not be involved in the differential viral infectivity in monocytes observed between the virulent and avirulent dengue-2 strains.

B. pH requirement for membrane fusion of virulent and avirulent dengue-2 viral strains.

Experiment were done to investigate if viral uncoating played a role in the variability in dengue-2 viral infectivity in monocytes. Viral membrane fusion is a crucial step during the viral uncoating that leads to infection of cells by enveloped viruses (Marsh and Helenius, 1989), including flaviviruses (Gollins and Porterfield, 1985; 1986a; 1986b). Therefore, viral membrane fusion activity of the virulent and avirulent strains of dengue-2 virus was examined.

Most enveloped viruses, including flaviviruses enter cells via the process called receptor mediated endocytosis during which viral virions are delivered into prelysosomal vacuoles called endosomes (Marsh, 1984; Marsh and Helenius, 1989). The acidic pH in these vacuoles apparently triggers a conformational change in the viral glycoprotein that initiates the fusion event between the viral membrane and the endosomal or lysosomal membrane. For certain viral systems, this fusion reaction is thought to allow the release of the viral genome or transcription complex into the cytosol. It is difficult to directly measure such fusion in the cell cytosol. However, several techniques have been developed to measure such activity externally by simulating the endosome-like condition at the outer cell-membrane. One of which is synonymous to an in vitro phenomenon is called "fusion-from-without". It occurs when virus particles are added to cells at a high MOI followed by lowering of medium pH to that found in endosomes. Fusion begins almost immediately and requires no synthesis of viral proteins, indicating that a viral structural component is involved (White et al., 1983). The optimal pH required for fusion varies from one virus to another (White et al., 1981).

It is plausible that the difference in pH optimum for viral membrane fusion may be related to infection-efficiency. This has been shown with a mutant of Semliki Forest Virus (SFV), which requires a lower pH for fusion, and exhibits inefficient infectivity (Kielien et al., 1984). These variations were shown to be related to uncoating of the wild type virus in the early endosome, on one hand, and of the mutant in late endosomes, on the other hand. Uncoating in the early endosome appeared to occur sooner after internalization and at a more rapid rate than that in the late endosome. (Kielien et al., 1986). Additional evidence with Newcastle Disease virus (NDV) suggested a relationship between viral fusion, efficiency of viral infectivity and viral virulence (Nagai et al., 1976; 1979). According to this paradigm for viral fusion, uncoating and infection, it is plausible that different fusion pH profiles, are related to variation in efficiency of viral uncoating and subsequent infection outcome. Thus, especially, a comparison of fusion pH profiles between dengue-2 the virulent and avirulent strains was done to generate further information that may relate to the biological basis of dengue-2 viral virulence.

To test the above hypothesis, the viral membrane fusion "from without" with dengue-2 virus was first examined. Cell-fusion was observed at a mildly acidic pH range (5.8-6.4) after the binding of the virus to the Ae. albopictus C6/36 cells at a high moi (> 100). The extent of fusion and the pH range of fusion activity varied among the strains tested (Figure 11). The virulent strain from Thailand exhibited fusion at the pH 6.1 - 6.7 while its avirulent counterpart exhibited fusion at a more restricted range of 6.2 - 6.5. The Puerto Rico virulent strain exhibited fusion at a more basic pH range of 6.2 - 6.7 as compared to that by the avirulent strain at 6.0 - 6.4. These data demonstrated slight differences in fusion pH profile between the virulent and avirulent strains that may affect monocyte

infectivity and hence the virulence of dengue-2 virus.

The above findings suggested that the fusion of dengue virus and cell membranes is likely to occur in vivo in an acidic compartment (i.e. endosomes). However, its role in the uncoating and the subsequent infection has not been established. Such a relationship between the acid-pH dependent fusion and infection can be preliminarily assessed by measuring the viral sensitivity to infection-inhibition by chloroquine, since chloroquine acts as an ionophore which prevents the lowering of pH in the endosomes and lysosomes. This compound thereby inhibits the acid-pH dependent viral membrane fusion event, resulting in inhibition of viral uncoating and thus infection.

The viral sensitivity to dengue infection-inhibition by chloroquine was evaluated with human monocytes and C6/36 mosquito cells. Viral infection in C6/36 mosquito cells was not affected by chloroquine at concentrations below cell toxicity , while inhibition was observed with human monocytes. In addition, difference in the sensitivity to chloroquine was observed between the virulent and avirulent pairs of dengue-2 virus. The virulent strain from Thailand and its avirulent counterpart exhibited sensitivity with the 50 % inhibition dose (ID_{50}) of 15 uM and 10 uM, respectively. The sensitivity ID_{50}s of the virulent strain from Puerto Rico and its avirulent counterpart to infection-inhibition by chloroquine were 9.5 uM and 7.0 uM respectively. Although slight, the higher sensitivity of both avirulent strains to infection-inhibition by chloroquine supported the earlier observation that the virulent dengue-2 strains required a more acidic environment for fusion with membranes of human monocytes than did the avirulent strains.

C. Reactivity of virulent and avirulent dengue-2 viral strains with monoclonal antibodies to dengue-2 E glycoprotein.

The viral envelope glycoprotein is the main viral component that is responsible for early stages of viral infection in most enveloped viruses. Variation on functionally specific regions of the envelope glycoproteins could lead to modification of the respective viral functions. Antigenic variations on the dengue envelope (E) glycoprotein has been reported among geographically different strains of dengue-2 virus (Monath et al., 1986). It is plausible that some of these modifications are related to the difference in viral infectivity to monocytes observed between dengue-2 virulent and avirulent strains. Mapping of these specific regions on the viral E glycoprotein with dengue specific monoclonal antibodies may lead to the identification of a well defined marker for dengue virulence.

First we determined whether there was antigenic variability on the E glycoprotein between dengue-2 strains that might correlate with virulence. This was accomplished by determining the hemagglutinating (HI), neutralizing and antibody dependent

enhancing (ADE) activities of the two pairs of dengue-2 strains with dengue-2 polyclonal antibody and four monoclonal antibodies that were reactive to dengue-2 E glycoprotein (Kaufmann et al., 1987). The results indicated differences in HI, $PRNT_{50}$, and ADE titers among the viral strains tested with certain monoclonal antibodies (Table 7). These findings suggested that there may be subtle antigenic differences on the E glycoprotein involved in these respective functional epitopes. An evaluation to determine whether these antigenic variations are relevant to virulence is essential for further identification of the virulence marker.

VII. Discussion

The present analysis indicated that the antibody-mediated infectivity of dengue-2 virus in human monocytes is a suitable *in vitro* correlate for virulence. This correlation for virulence may also apply to dengue serotype-4 but not dengue serotypes -1 and -3. Such a lack of association between monocyte infectivity and the severity of dengue illness by the latter two serotypes may explain the different disease patterns associated with dengue-1 and dengue-3 viruses. For example, an equal incidence rate of DHF/DSS was observed with dengue-1 virus among primary and secondary cases (Burke et al., 1988). In addition, a relatively high incidence of DHF/DSS cases in primary cases was observed with dengue-3 (Hoke et al., 1985). Thus, it appears that severe illnesses associated with dengue-1 and dengue-3 viruses may have a divergent pathogenetic mechanism from dengue-2 and dengue-4 viruses. Additional detailed epidemiological, virological and clinical evaluation on dengue-1 and dengue-3 viruses is required to gain further evidence for this apparent difference in the disease pathogenesis.

The validity of monocyte infectivity as an *in vitro* correlate for virulence of dengue-2 virus is supported by the observation that dengue-2 viral strains with high monocyte infectivity were found in topotypes unique to the DHF/DSS prevalent areas; while dengue-2 viral strains with low monocyte infectivity were found in all dengue endemic areas (Figure 3). However, our results indicated that many strains with the infectivity indicies in the middle range were associated with both PUO/DF and DHF/DSS (Table 3). Thus, this biological property which involves multiple steps of events may not serve as an effective marker for viral virulence and therefore further search for a more well defined marker for dengue virulence is required.

Our analysis on the early events leading to viral infection in monocytes indicated that the viral uncoating rather than viral attachment or internalization may be related to the differential infectivity observed between virulent and avirulent strains of dengue-2 virus. This difference in infectivity, however, was not observed in the viral infection of *Ae. albopictus* C6/36 cells indicating an involvement of host cell factor(s).

Lack of difference in viral binding capacity between the virulent and avirulent dengue-2 viruses to both monocytes and C6/36 cells suggests that the viral function important to the maintenance of its life cycle is not affected by the variation of virulence. Thus, viral uncoating may serve as a step that modulates viral infection in a specific target cell, and in turn, plays a role in the pathogenesis in the human host without compromising the maintenance of the virus in the mosquito host.

It is plausible that the subtle differences observed in two topotypic pairs of virulent and avirulent dengue-2 viruses regarding the pH fusion profiles is related to the antigenic variation observed on the viral E glycoprotein. If so, the variation in amino acid sequence of the fusion peptide on the viral E glycoprotein may affect viral infectivity and hence, viral virulence. Such model has been demonstrated in influenza virus (Webster and Rott, 1987) and NDV (Nagai et al, 1976;1979; Toyoda et al, 1987). To determine if the same basis for virulence is applicable to dengue-2 virus, the relationship among viral membrane fusion at a higher pH, a higher efficiency of viral uncoating and the infection outcome should be established. Finally, the determination of genomic sequence variation at the fusion region of the viral E glycoprotein may be an appropriate step toward the identification of a molecular virulence marker for dengue-2 virus.

Table 1. Variables included in the correlation analyses

1. TEST NUMBER: represents each monocyte donor (NIH blood bank).
2. CODE: represents each viral isolate previously coded by CDC.
3. LOCATION: origin of viral isolates.
4. EPIDEMIOLOGICAL STATUS: 1 = no DHF
   2 = sporadic DHF
   3 = epidemic DHF
5. CLINICAL STATUS: 0 = PUO or DF
   1 = DHF/DSS
6. MONOCYTE INFECTION WITHOUT AB: % FA positive monocytes.
7. MONOCYTE INFECTION WITH ENHANCING AB: % FA positive monocytes.
8. FA PATTERN: 1 = focal
   2 = granular
   3 = complete
9. ENHANCED MONOCYTE INFECTION: increase of % FA positive monocytes. (#7-#6)
10. VIRUS YIELD WITHOUT AB: Log_{10} PFU/ ml.
11. VIRUS YIELD WITH ENHANCING AB: Log_{10} PFU/ ml.
12. ENHANCED VIRUS YIELD: increase of virus yield in Log_{10} PFU/ml (#11-#10)

Table 2. Correlation between DHF/DSS outcome in patient and monocyte infection parameters of the infecting virus

	Pearson		Spearman		Logistic Regression	
	r	p	r	p	p increment per unit increment	p
VYAb@	0.476	<0.001	0.482	<0.001	32 %	<0.001
ENVY*	0.366	<0.001	0.282	<0.05	15 %	<0.01
IFA#	0.409	<0.001	0.287	<0.05	20 %	<0.01

72 Observations

r = correlation coefficient
p = probability
@ virus yield in the presence of enhancing antibodies
* enhanced virus yield
# FA staining pattern

Footnotes: Clinst. = clinical status, 0 = DF and 1 = DHF/DSS, 999 = unknown and therefore omitted from the analysis.
VYAB = virus yield in the presence of enhancing antibodies
ADJVYAB = virus yield (in the presence of enhancing antibodies) adjusted against the reference virus strain 16681 in each experiment = 100.

Table 3. Dengue-2 viral isolates ranked from high to low monocyte-infectivity

A

CODE	CLINST	VYAB		LOCATION
680	1	7.3		THAI
124	999	6.6		JAMAICA
37	1	6.5		THAI
565	1	6.4		THAI
828	1	6.2		THAI
381	1	6.2	100%	THAI
990	1	6.2		THAI
973	1	6.2		THAI
670	1	6.1		THAI
529	1	6		THAI
982	1	5.8		THAI
62	1	5.6		THAI
133	0	5.5		PR
16681	1	5.5	94%	THAI
460	1	5.3		THAI
742	1	5.3		PR
658	1	5.2		PHIL
293	1	5		THAI
801	0	4.9		TRINIDAD
1256	0	4.9		INDO
1013	1	4.8		INDO
729	1	4.7		PHIL
294	0	4.5		THAI
782	0	4.5		SRI
491	0	4.5		FIJI
737	1	4.4		THAI
884	1	4.4		THAI
658	1	4.4		PHIL
1208	1	4.4		INDO
669	0	4.2		PR
728	0	4.2		PR
519	1	4.2		PHIL
10099	1	4.2		PHIL
4	0	4.2		FIJI
629	0	4.1	68%	SRI LANKA
616	1	4		THAI
654	0	4		PHIL
463	1	4		PHIL
745	1	3.9		THAI
1016	1	3.9		INDO
1209	0	3.8		INDO
40979	1	3.7		BURMA
399	1	3.6		THAI
42	1	3.6		INDO
46	999	3.6		BURMA
202	0	3.5		PR
34	1	3.5		INDO
144	999	3.4		TRINIDAD
483	0	3.4		PHIL
71	0	3.4		FIJI
419	999	3.15		TRINIDAD
22	0	3.1		BURMA
742	1	3		PR
1261	1	3		INDO
1122	1	3		INDO
54	1	3		DOMREP
153	1	2.9		THAI
32	0	2.9		TAHITI
731	0	2.9		PHIL
263	0	2.8		THAI
220	999	2.8		INDO
655	1	2.8		INDO
889	1	2.6		PHIL
525	0	2.5		FIJI
254	1	2.3		PR
555	0	2.2		PR
872	0	2.2		JAMAICA
160	0	1.8		PR
975	0	1.7		SRI LANKA
860	0	1.7		PR
411	0	1.7		PR
44	0	1.6		MEX
718	0	0.7		TONGA
16	0	0.7		TAHITI
32	0	0.7		TAHITI
563	0	0.7		MEXICO
565	0	0.7		FIJI

B

CODE	CLINSTAT	ADJVYAB		LOCATION
990	1	135		THAI
62	1	122		THAI
124	999	122		JAMAICA
742	1	115		PR
658	1	113		PHIL
680	1	106		THAI
1261	1	106		INDO
293	1	100		THAI
16681	1	100		THAI
801	0	96		TRINIDAD
37	1	94		THAI
565	1	93		THAI
973	1	92	94%	THAI
381	1	90		THAI
828	1	90		THAI
737	1	88		THAI
670	1	88		THAI
529	1	87		THAI
745	1	85		THAI
982	1	84		THAI
294	0	83		THAI
616	1	80		THAI
463	1	80		PHIL
133	0	80		PR
519	1	78		PHIL
460	1	77		THAI
629	0	76		SRI LANKA
34	1	76		INDO
1013	1	72		INDO
42	1	72		INDO
46	999	70	93%	BURMA
729	1	70		PHIL
884	1	68		THAI
399	1	67		THAI
491	0	67		FIJI
1208	1	66		INDO
782	0	65		SRI LANKA
658	1	64		PHIL
144	999	63		TRINIDAD
4	0	63		FIJI
669	0	62		PR
728	0	62		PR
10099	1	62		PHIL
22	0	61		BURMA
220	999	61		INDO
654	0	60		PHIL
742	1	60		PR
419	999	58		TRINIDAD
1016	1	58		INDO
1209	0	57		INDO
655	1	56		INDO
40979	1	55		BURMA
32	0	54		TAHITI
153	1	54		THAI
263	0	52		THAI
202	0	52		PR
71	0	51		FIJI
483	0	49		PHIL
889	1	48		PHIL
525	0	46		FIJI
1256	0	45		INDO
1122	1	45		INDO
254	0	45		PR
54	1	45		DOMREP
872	0	44		JAMAICA
555	0	44		PR
731	0	43		PHIL
975	0	37		SRI
160	0	36		PR
860	0	33		PR
411	0	33		PR
44	0	26		MEX
32	0	15		TAHITI
563	0	14		MEXICO
718	0	14		TONGA
16	0	14		TAHITI
565	0	14		FIJI

Table 4. Correlation between antibody-mediated monocyte infectivity of the virus and the severity of illness by dengue-2 and -4 but not dengue-1 and -3 viruses.

	Den-1	Den-2	Den-3	Den-4
Number of strains (n)	41	72	27	19
Correlation coef.*(r)	-.204	.476	-.321	.425
p value	nf	<.001	nf	<.05

Table 5. Comparison of infectivity of dengue-2 virus in K-562 monocytes and viral infectivity index derived from infection of primary human monocytes.

Dengue-2 strains	Mo infect. index	Infection without enhc. Ab		Infection with enhc. Ab	
		EIA O.D.	Log_{10} PFU/ml	EIA O.D.	Log_{10} PFU/ml
Thailand-64 565	122	1.061	5.20	1.107	5.34
Thailand 680	106	0.051	4.15	0.473	6.32
Thailand-64 16681	100	0.869	5.02	1.057	6.36
Thailand-80 293	100	0.036	3.42	0.860	5.68
Thailand-81 973	92	1.221	4.20	1.640	5.58
Trinidad-82 801	96	0.497	3.40	1.076	4.41
Sri Lanka 629	76	0.349	3.40	1.232	5.14
Fiji-71 491	67	0.102	2.40	1.311	3.41
Burma 22	61	0.022	3.25	0.607	5.00
Burma D-46	55	0.024	2.90	0.107	4.98
Thailand-80 263	52	0.452	2.60	0.702	4.38
Indonesia- 1256	45	0.390	3.60	0.643	5.14
Puerto Rico-86 411	33	0.104	2.08	1.173	3.48
Mexico-83 044	26	0.154	2.70	1.116	4.08
Mexico-86 086	14	0.013	0.07	0.935	3.00
R		.54	.82	.14	.75
p		<.01	<.0005	not sig.	<.0005

# Infection was performed at MOI 0.1
R = Correlation coefficient to Mo (monocyte) infectivity indices

Table 6. Comparison of K-562 and CZ monocytic cell lines for susceptibility to selected strains of dengue-2 virus.

Viral strains	Mo Infect. Index	K-562 cells		CZ cells	
		EIA O.D.	Log_{10} PFU/ml	O.D.	Log_{10} PFU/ml
Thailand 062	122	0.718	5.79	0.780	2.25
Thailand 16681	100	0.635	6.11	0.560	4.60
Thailand 680	106	0.994	5.25	0.953	2.32
Thailand 670	88	0.042	3.60	0.452	2.50
Puerto Rico 133	80	0.437	2.78	0.367	0.60
Indonesia 1209	57	0.304	2.25	0.289	2.08
Philippines 483	49	1.067	4.45	0.829	1.93
Puerto Rico 860	33	0.012	1.50	0.000	0.03
R		.41	.76	.52	.73
p values			<.01		<.01

R = Correlation coefficient to the Mo (monocyte) infectivity indices
# Infection performed at MOI 0.05 without enhancing antibodies.

Table 7. Variation among virulent and avirulent strains of dengue-2 virus in their reactivity with the E-glycoprotein reactive monoclonal antibodies.

Test	Mabs	Specificity	Thailand A 293	Thailand V 263	Puerto A 742	Puerto V 160
HI	4E5-6	subgroup	80@	320	80	160
	3H5	type	2560	5120	640	640
	4G2	group	10240	10240	1280	1280
	D_2HMAF*		640	1280	320	320
$PRNT_{50}$	4E5-6	subgroup	1300#	1200	4000	4500
	3H5	type	640	900	8200	14,000
	4G2	group	3500	8000	12,000	10,000
	D_2HMAF*		3800	6200	10,000	18,000
pADE	4E5-6	subgroup	100**	1000	1000	1000
	3H5	type	1000	10,000	1000	10,000
	4G2	group	10,000	10,000	100	1000
	D_2HMAF*		5000	5000	5000	5000

Mabs = monoclonal antibodies

A = avirulent; V = virulent

* = dengue-2 hyperimmune mouse ascitic fluid

@ = hemagglutination inhibition titer

= fifty percent plaque reduction neutralizing titer

** = peak antibody dependent enhancing titer

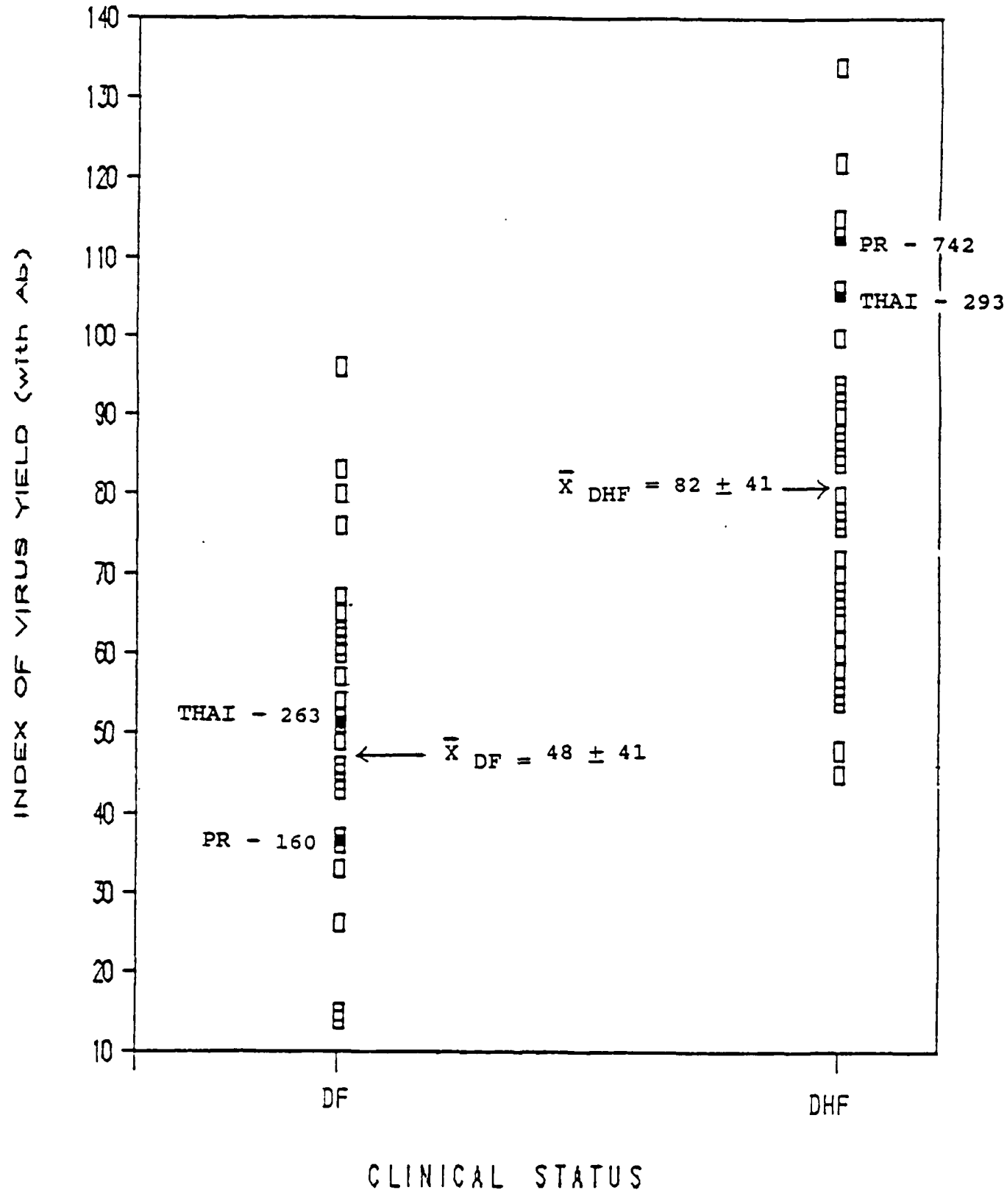

Figure 1. Difference in monocyte-infectivity between dengue-2 viral isolates associated with DF and with DHF (range and mean + 2 S.D.)

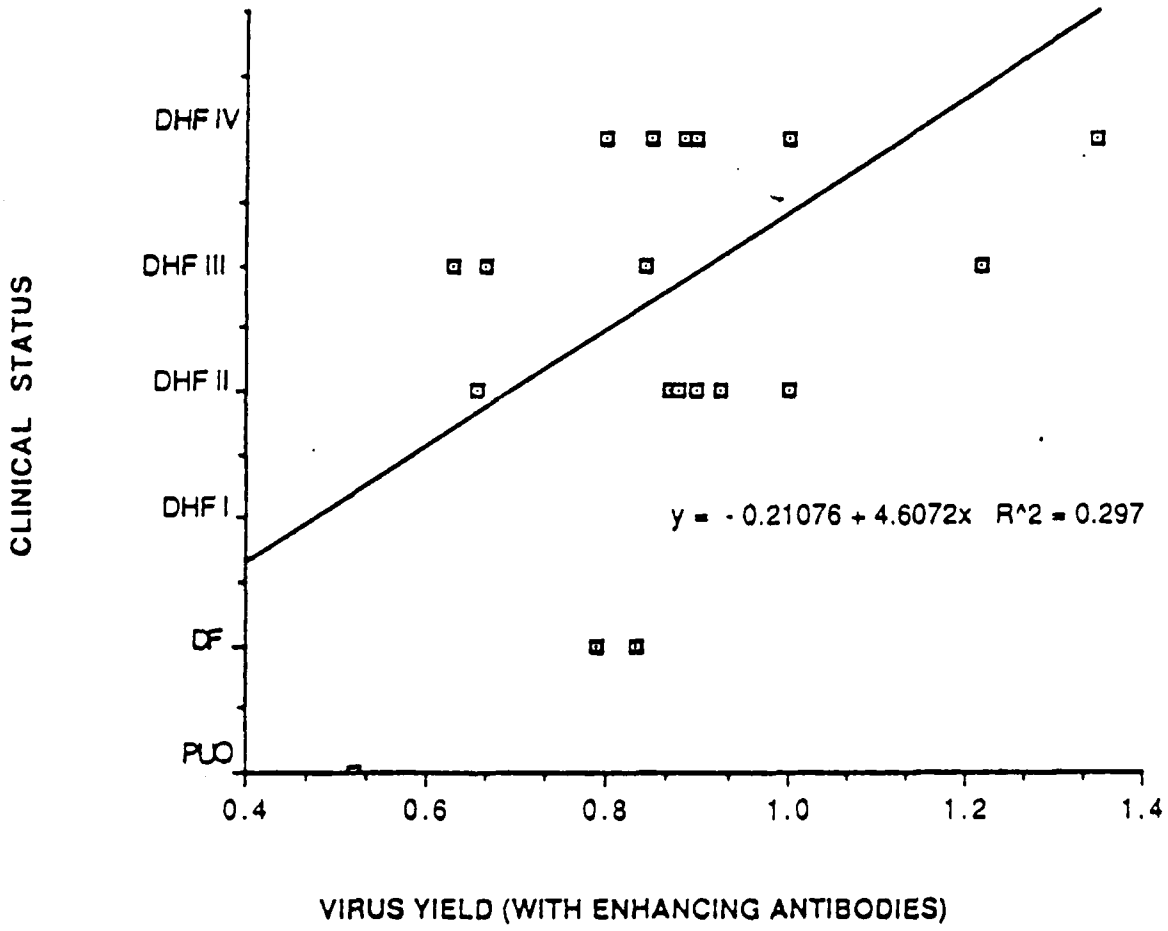

Figure 2. Correlation between the severity of illness associated with dengue-2 viral isolates from Thailand during 1970-84 and the antibody-mediated viral infectivity as measured by virus yields. The degree of disease severity ranges from mild (PUO = pyrexia of unknown origin) to the most severe (DHF IV = DSS).

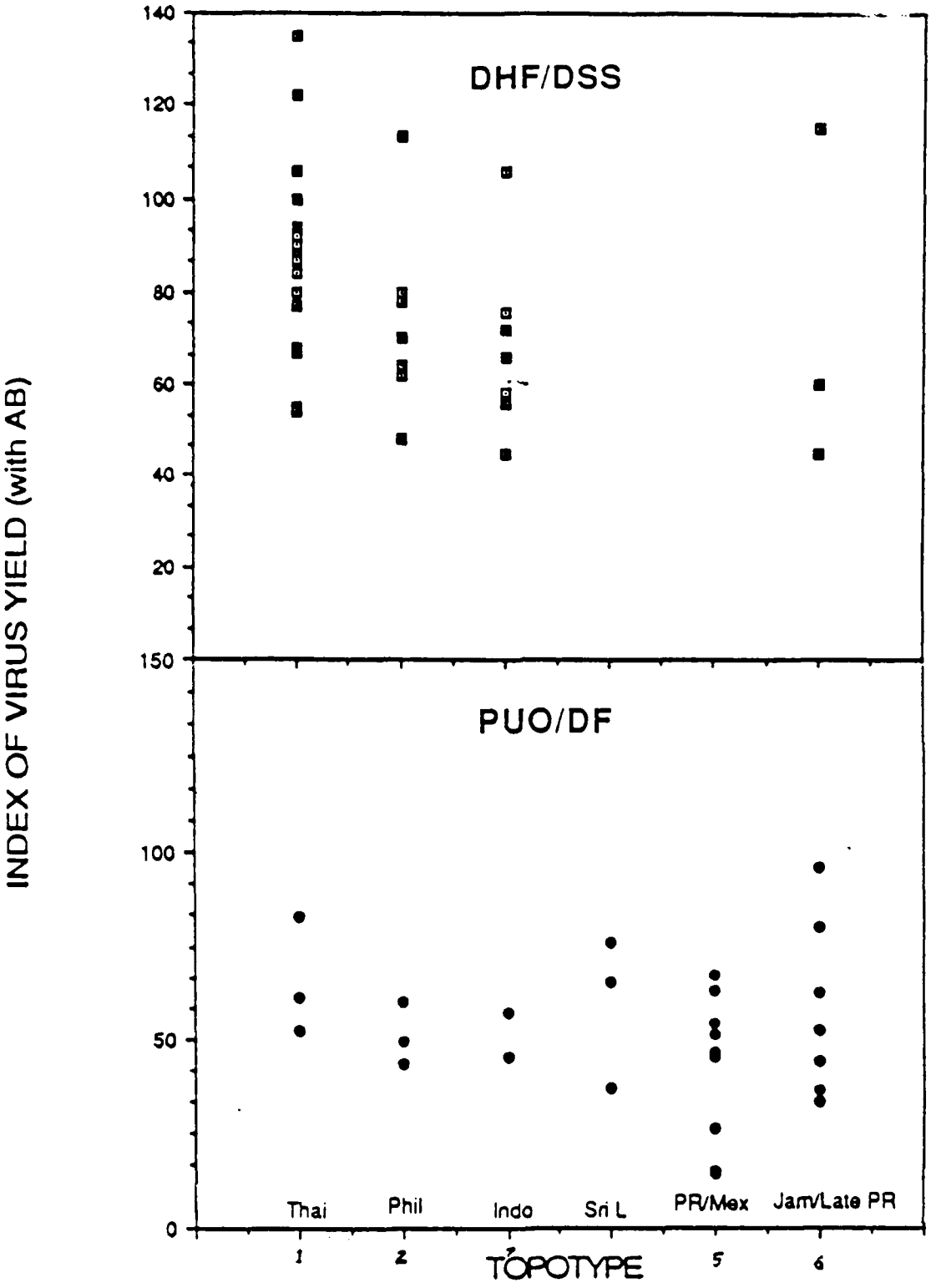

Figure 3. Distribution of dengue-2 viral isolates from PUO/DF and DHF/DSS patients among different topotypes. 1 = Thailand/Burma, 2 = the Philippines, 3 = Indonesia, 4 = Sri lanka, 5 = Early Puerto Rico/ Mexico and 6 = Jamaica/late Puerto Rico. Squares (◘) represent viral isolates associated with DHF/DSS, circles (●) represent viral isolates associated with PUO/DF.

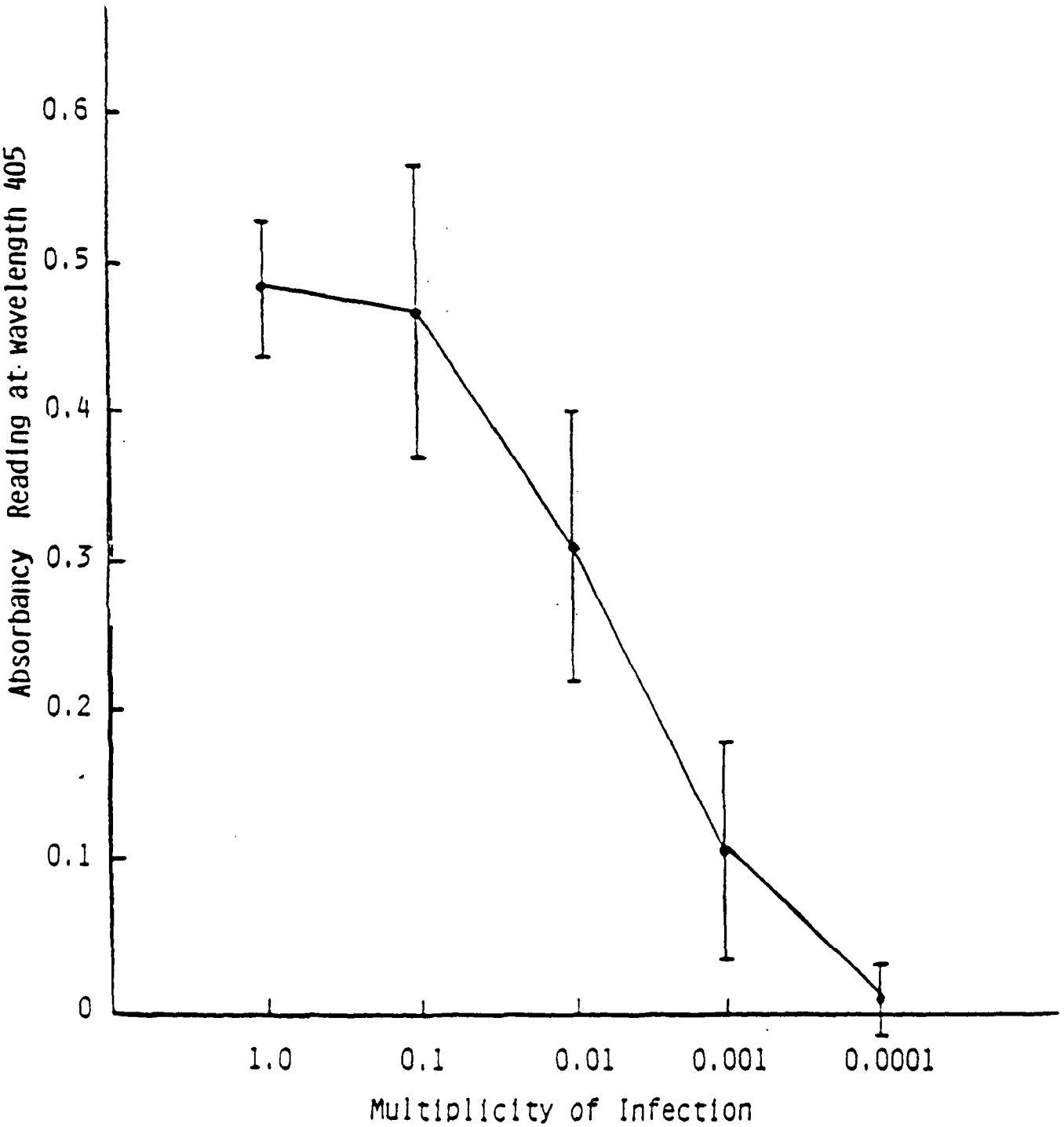

Figure 4. Dengue-2 viral infection of BHK-21 cells at various MOI as measured by the in situ EIA after 72 hrs incubation at 36°C . (Each data point represents a mean value from three experiments and the bars represent one standard error).

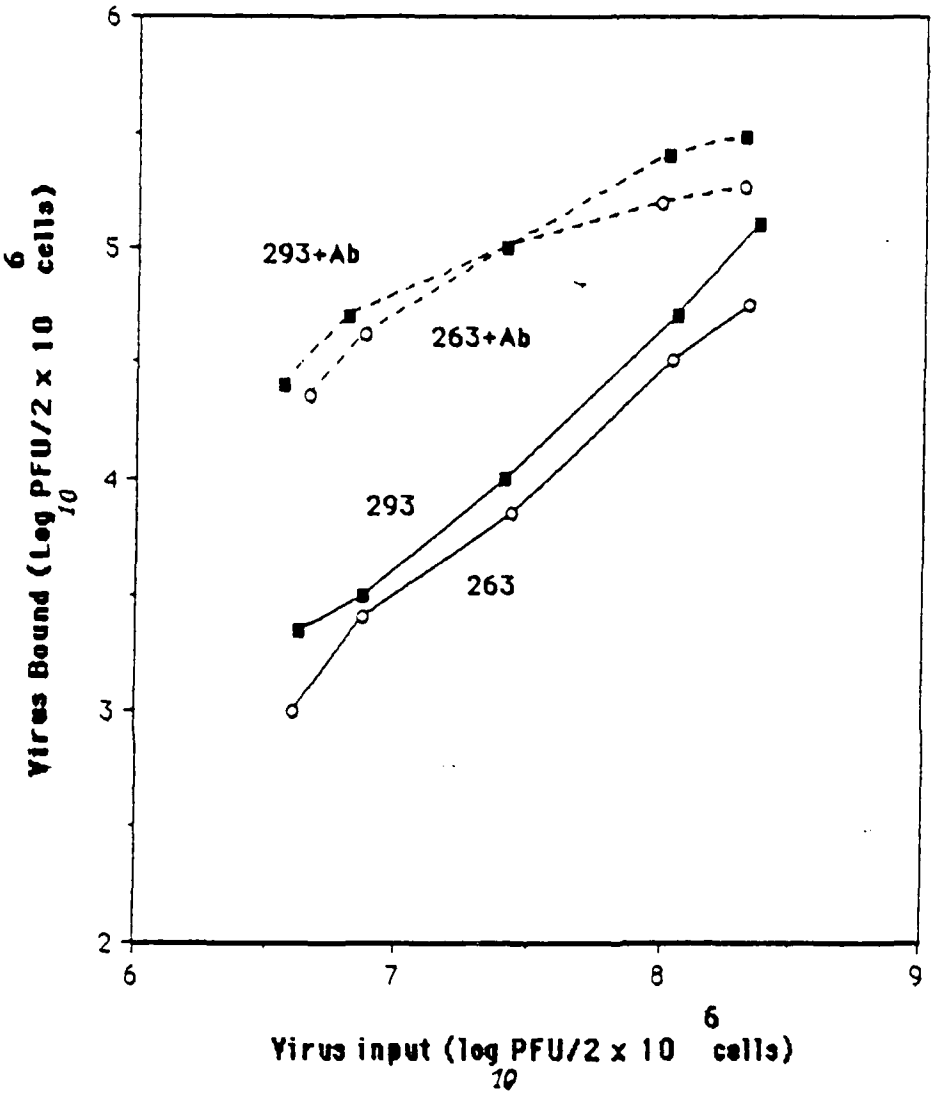

Figure 5. Binding of dengue-2 viral virulent, Thai 293 strain (■) and avirulent Thai 263 strain (o) to human monocytes, without enhancing antibodies (——) and with enhancing antibodies (-----). Ab = Enhancing dengue polyclonal antibodies.

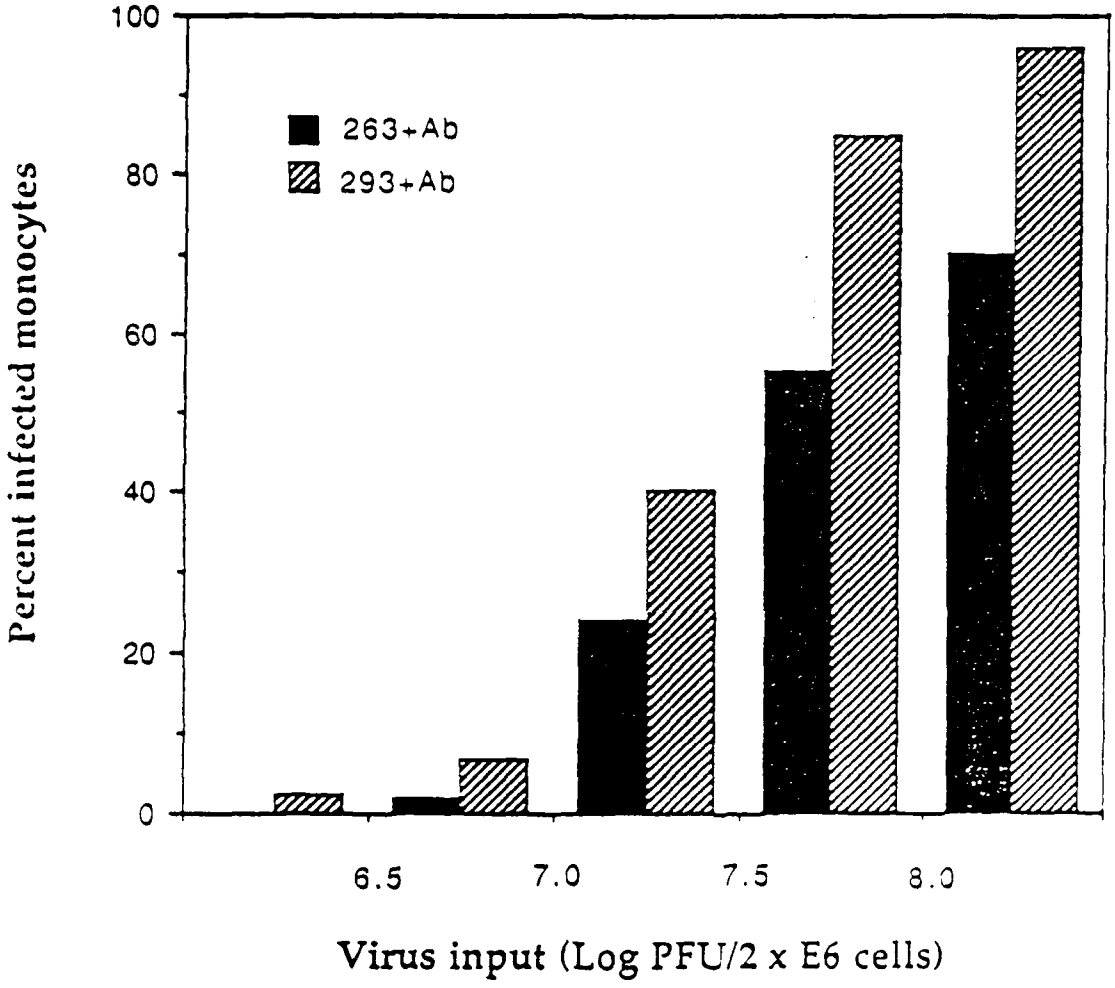

Figure 6. Difference in antibody-mediated infection levels in monocytes between the Thai virulent strain (■) and the Thai avirulent strain (▨) as expressed in percent infected monocytes.

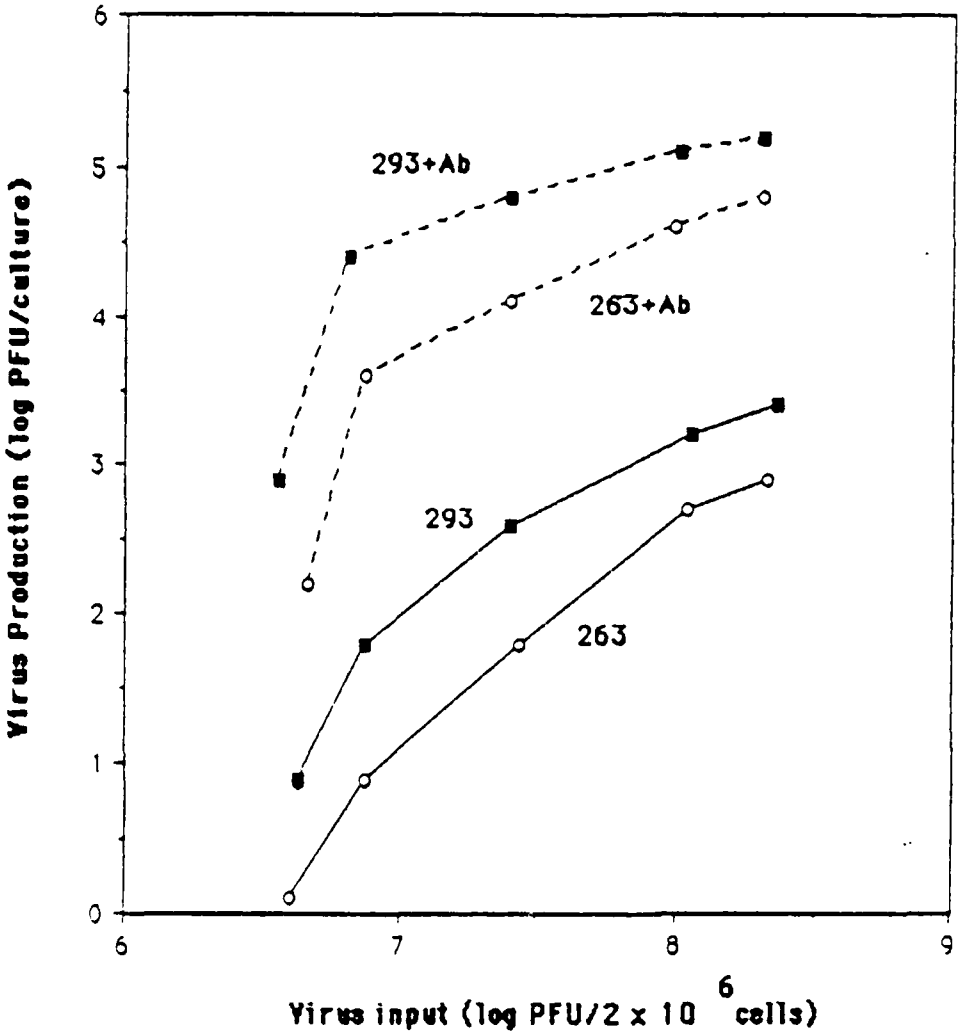

Figure 7. Difference in normal (——) and antibody-mediated (----) infection levels in monocytes between virulent Thai 293 strain (■) and avirulent Thai 263 strain (○) as expressed in virus yields. Ab = Enhancing dengue polyclonal antibodies.

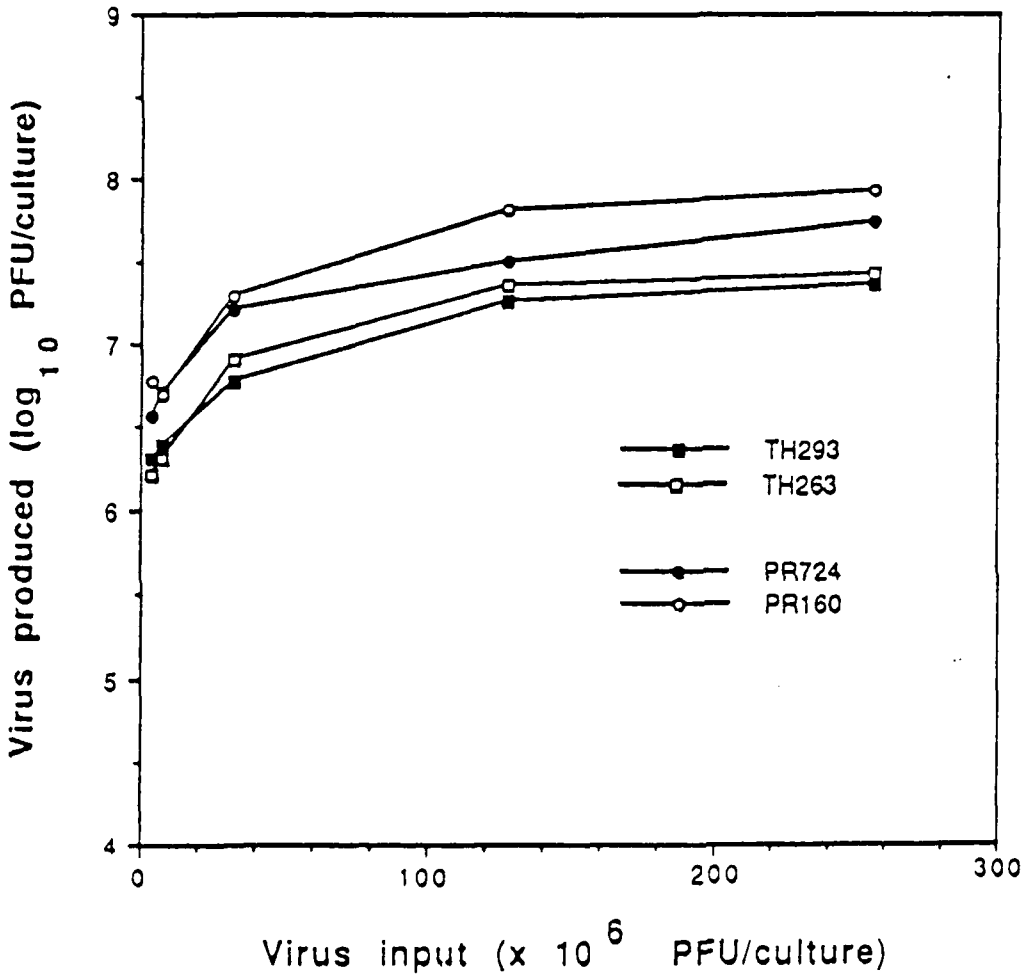

Figure 8. Similarity in binding capacities of the two virulent strains, Thai 293 (■) and Puerto Rico 724 (●) and two avirulent strains, Thai 263 (□) and Puerto Rico 160 (○), of dengue-2 virus to Ae. albopictus C6/36 cells.

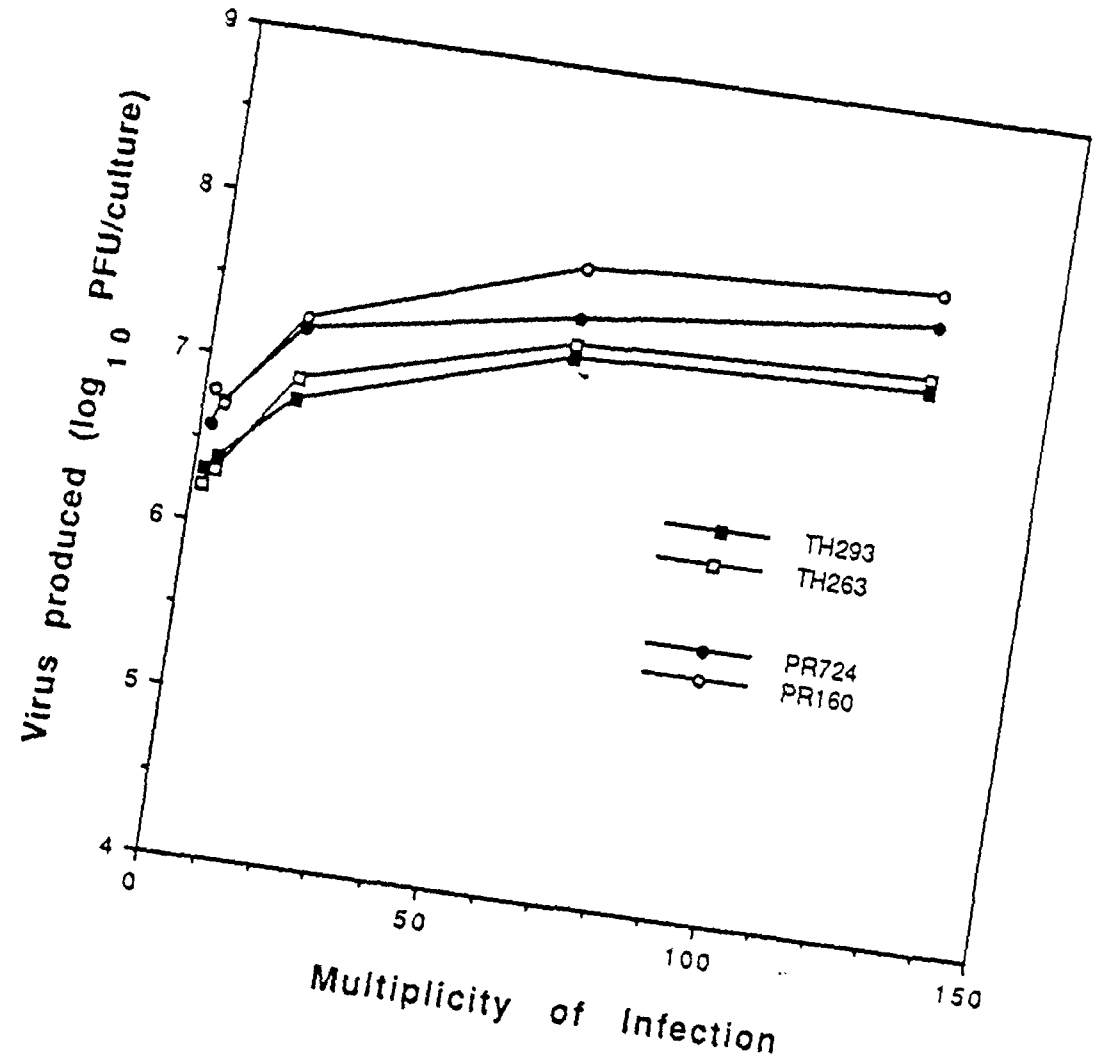

Figure 9. Similarity in the titers of the two virulent strains, Thai 293 (■) and Puerto Rico 724 (●) and two avirulent strains, Thai 263 (□) and Puerto Rico 160 (o) of dengue-2 virus produced in C6/36 Ae. albopictus cells.

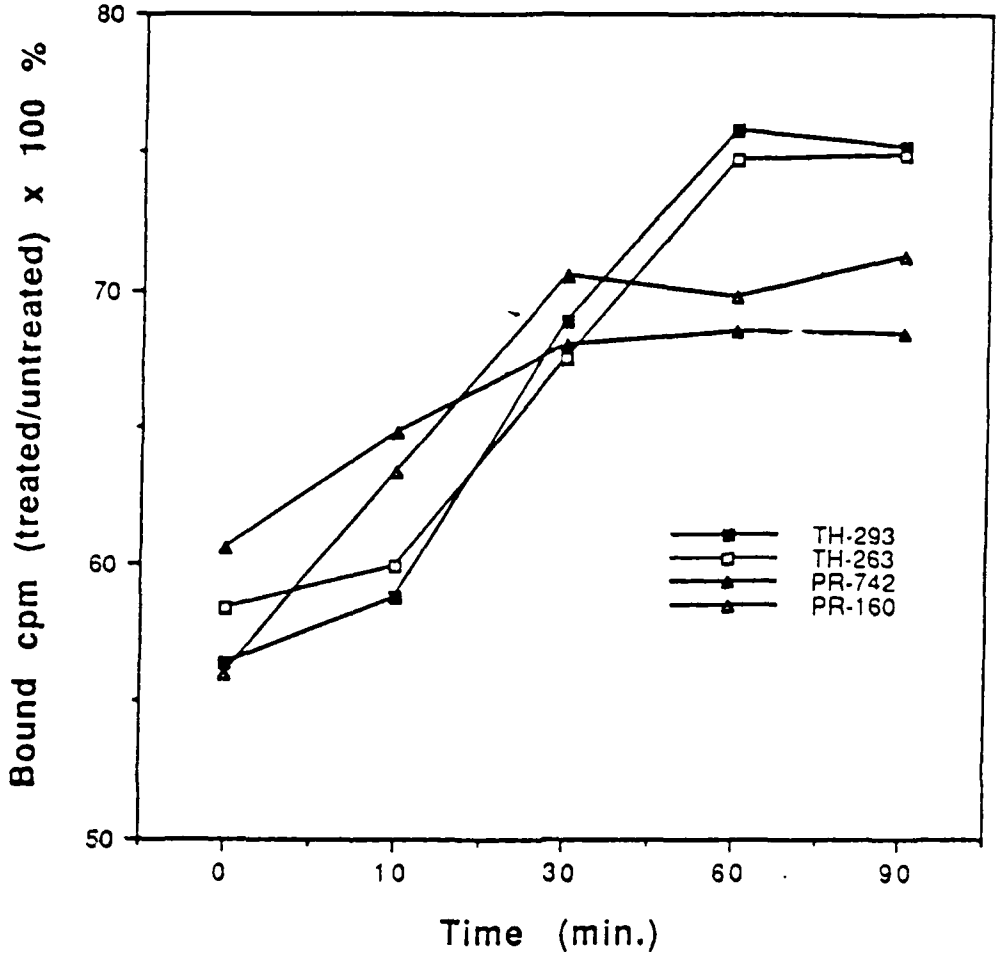

Figure 10. Similarity in the relative rate of internalization of the Thai virulent 293 (■) and avirulent 263 (□) strains and Puerto Rico virulent 742 (▲) and avirulent 160 (△) strains of dengue-2 virus into primary human monocytes.

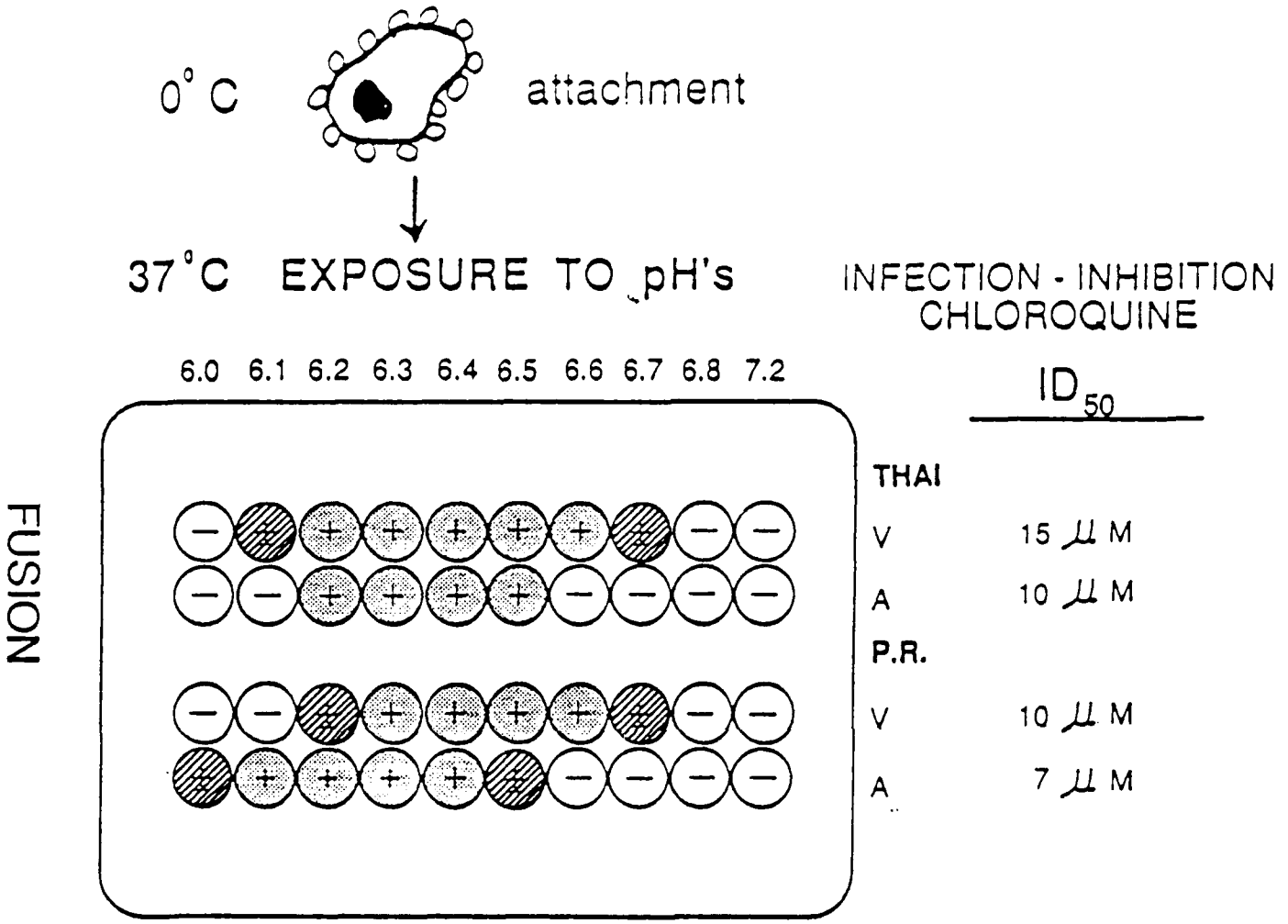

Figure 11. Schematic diagram indicating difference in pH membrane fusion profile between the two pairs of virulent and avirulent strains of dengue-2 virus as indicated by the fusion with C6/36 Ae. albopictus cell monolayers and by the inhibition of viral infection in human monocytes by chloroquine.

VIII. Literature cited

Bravo, J.R., Guzman M.G. and Kouri G.P. 1987. Why dengue haemorrhagic fever in Cuba? 1. Individual risk factors for dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). Trans. Roy. Soc. Trop. Med. & Hyg. 81: 816-820.

Burke, D.S., Nisalak, A., Johnson, D.E. and Scott R.McN. 1988. A prospective study of dengue infections in Bangkok. Am.J. Trop.Med.Hyg., 38: 172-180.

Dengue surveillance summary No. 51, 1988. Edited by San Juan Laboratories, Dengue branch, Division of Vector-borne Viral Diseases, CDC. P.O.Box 4532, San Juan, PR 00936.

Eram, S., Setyabudi,V., Sadono,T.I., Sutrisno, D.S., Gubler, D.J. and Saroso, J.S. 1979. Epidemic dengue hemorrhagic fever in Rural Indonesia. Am.J. Trop. Med. Hyg. 27: 711-716.

Gollins, S.W. and Porterfield, J.S. 1985. Flavivirus infection enhancement in macrophages: an electron microscopic study of viral cellular entry. J. Gen. Virol. 66:1969-1982.

Gollins, S.W. and Porterfield, J.S. 1986a. pH-dependant fusion between the flavivirus West Nile and liposomal model membranes. J. Gen. Virol. 67:157-166.

Gollins, S.W. and Porterfield, J.S. 1986b. The uncoating and infectivity of the flavivirus West Nile on interaction with cells: Effects of pH and Ammonium Chloride. J. Gen. Virol.67: 1941-1950.

Gubler, D.J., Suharyano, W., Lubis, I., Eram, E. and Sulianto Saroso, J. 1979. Epidemic dengue hemorrhagic fever in rural Indonesia. I. Virological and epidemiological studies. Am. J. Trop. Med. & Hyg. 28: 701-710.

Guzman, M.G., Kouri, G.P., Bravo, J., Calunga, M., Soler, M., Vasquez, S., and Venero, C. 1984. Dengue hemorrhagic fever in Cuba. I. Serologic confirmation of clinical diagnosis. Trans.Roy.Soc.Trop. Med.Hyg. 78: 235-238.

Halstead, S.B., Nimmanitya, S., and Cohen, S.N. 1970. Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of the disease severity to antibody response and virus recovered. Yale J. Biol. Med., 42: 311-328.

Halstead,S.B., O'Rourke, E.J. and Allison, A.C. 1977. Dengue virus and mononuclear phagocytes. II. Identity of blood and tissue leukocytes supporting in vitro infection. J. Exp.Med. 146: 218-229.

Halstead, S.B., 1984. The pathogenesis of dengue. Molecular epidemiology of infectious disease. Am. J. Epidemiol., 144: 632-648.

Halstead, S.B., C.N. Venkateshan, M.K. Gentry and L.K. Larsen. 1984. Heterogeniety of infection enhancement of dengue-2 strains by monoclonal antibodies. J. Immunol. 132: 1529-1532.

Hoke, C., Nisalak, A., and Burke, D.S. 1983. Dengue serotypes isolated from hemorrhagic fever patients at Bangkok Children's hospital, 1962-1983. Pages 166-178 in T. Pang and R. Pathmanathan, eds., Proceedings of the International Conference on Dengue Hemorrhagic Fever. University of Malaya, Kuala Lumpur, Malaysia.

Kielian, M., Keranen, M.C., Kaariainen, S., et al. 1984. Membrane fusion mutants of Semliki Forest virus. J. Cell Biol. 98:139-145.

Kielien, M.C. Kielian, M. Marsh and A. Helenius. 1986. Kinetics of endosome acidification detected by mutant and wild type Semliki Forest virus. EMBO J. 5: 3103-3109.

Kliks, S.C., Nimmanitya, N., Nisalak, A. and Burke, D.S. 1988. Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. 1988. Am. J. Trop. Med. Hyg. 38:411-419.

Kliks, S.C., Nisalak, A., Brandt W.E. and Burke, D.S. 1989. Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever.Am. J. Trop. Med. Hyg. 40: 140-144.

Kliks, S.C., A. Nisalak, M.S. Seguin and D.S. Burke. Interserotypic difference in virulence of dengue virus: In vitro model (submitted).

Kouri, G.P., Guzman, M.G. and Bravo, J.R. 1987. Why dengue haemorrhagic fever in Cuba? 2. An integral analysis. Roy. Soc. Trop. Med. Hyg. 81: 821-823.

Kuafmann B.M., Summers, P.L., Dubois D.R. and Eckels, K.H. 1987 Monoclonal antibodies against dengue-2 virus E-glycoprotein protects mice against lethal dengue infection. Am. J. Trop. Med. Hyg. 36:427-434.

Lozzio B.B. and Lozzio, C.B. 1979. Properties and usefulness of the original K-562 human myelogenous leukemia cell line. Leuk. Res. 3:363-364.

Marsh M. and Helenius, A. 1989. Virus entry into animal cells. Avd. in Vir. Res. 36:107-151.

Marsh, M. 1984. The entry of enveloped viruses into cells by endocytosis. Biochem J. 218:1-10.

Monath, T.P., Wands, J.R., Hill, L.J. et al., 1986. Geographic classification of dengue-2 virus strains by antigen signature analysis. Virology. 154:313-324.

Morens, D.M., Larsen, L.K. and Halstead, S.B. 1987. Study of the distribution of antibody-dependent enhancement determinants on dengue-2 isolates using dengue-2 derived monoclonal antibodies. J. Med. Virol, 22: 163-167.

Nagai, Y., Klenk H.D. and Rott, R. 1976. Proteolytic cleavage in the viral glycoproteins and its significance for the virulence of Newcastle disease virus. Virology 72:494-508.

Nagai, Y., Shimokata. K, Yoshida, T., et al. 1979. The spread of a pathogenic and an apathogenic strain of Newcastle disease virus in the chick embryo as depending on the protease sensitivity of the virus glycoproteins. J. Gen. Virol. 45:263-272.

Repik, P.M., Dalrymple, J.M., Brandt, W.E., McCown, J.M and Russell, P.K.1983. RNA finger printing as a method for distinguishing dengue-1 virus strains. Amer. J. Trop. Med. Hyg. 32:577-589.

Rosen, L. 1977. The emperor's new clothes revisited, or reflections on the pathogenesis of dengue hemorrhagic fever. Am. J. Trop. Med. Hyg. 26: 337-343.

Toyoda T, Sakaguchi, T., Imai, K., et al. 1987. Structural Comparison of the cleavage-activation site of the fusion glycoprotein between virulent and avirulent strains of Newcastle Disease Virus. Virology 158: 242-247.

Trent, D.W., Grant, J.A., Rosen, L. and Monath, T.P. 1983. Genetic variation among dengue-2 viruses of different geographic origin. Virology. 128: 271-284

Wahl, L.M., Katona, R.L., Wilder, R.L., Wilder, C.C., Haranoui, I., Scher, R. and Wahl, S.M. 1984. Separation of human mononuclear cells by centrifugal elutriation. J. Cell. Immunol. 85: 373-383.

Webster , R.G. and Rott, R. 1987. Influenza virus A Pathogenicity: The Pivotal Role of Hemagglutinin. Cell 50: 665-666.

White, J., Matlin, K. and Helenius, A. 1981. Cell fusion by Semliki Forest, Influenza, and Vesicular Stomatitis viruses. J. Cell Biol. 89: 647-679.

White, J., Kielian, M. and Helenius, A. 1983. Membrane fusion proteins of enveloped animal viruses. Quarterly Rev. Biophysics 16:151-195.

Yong-He, Z., Fang, Y.W., Wen, T.Z., Qian, G.J., Sheng, C.Q. and Min, W.Y. 1984. A simplified new method for the identification of arboviruses: virus detection using enzyme immunoassay on cultured cells. J. Virol. Meth. 9: 45-51.

DISTRIBUTION LIST

4 copies	Director Walter Reed Army Institute of Research ATTN: SGRD-UWZ-C Washington, DC 20307-5100
1 copy	Commander US Army Medical Research and Development Command ATTN: SGRD-RMI-S Fort Detrick, Frederick, Maryland 21701-5012
2 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100