

SECURITY

FILE COPY

1

AD-A200 828

MENTATION PAGE

Form Approved
OMB No 0704 0188
Exp Date Jun 30, 1986

1a. 1 UNCLASSIFIED		1b. RESTRICTIVE MARKINGS	
2a. SECURITY CLASSIFICATION AUTHORITY UNCLASSIFIED		3. DISTRIBUTION/AVAILABILITY OF REPORT	
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE UNCLASSIFIED			
4. PERFORMING ORGANIZATION REPORT NUMBER(S)		5. MONITORING ORGANIZATION REPORT NUMBER(S)	
6a. NAME OF PERFORMING ORGANIZATION AFRIMS	6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION Walter Reed Army Inst. of Rsch.	
6c. ADDRESS (City, State, and ZIP Code) Washington, DC 20307-5100		7b. ADDRESS (City, State, and ZIP Code) Washington, DC 20307-5100	
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Ft Detrick, Frederick, MD	8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8c. ADDRESS (City, State, and ZIP Code) US Army Medical Res & Dev Command Ft Detrick, Frederick, MD 21701-5012		10. SOURCE OF FUNDING NUMBERS	
		PROGRAM ELEMENT NO.	PROJECT NO.
		TASK NO.	WORK UNIT ACCESSION NO.
11. TITLE (Include Security Classification) DETECTION OF FLAVIVIRUS ANTIBODIES IN HUMAN SERUM BY EPITOPE-BLOCKING IMMUNOASSAY			
12. PERSONAL AUTHOR(S) D.S. BURKE, A. NISALAK, AND M.K. GENTRY			
13a. TYPE OF REPORT Manuscript	13b. TIME COVERED FROM _____ TO _____	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
1. SUPPLEMENTARY NOTATION			
17. COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	SUB-GROUP	
19. ABSTRACT (Continue on reverse if necessary and identify by block number)			
DTIC ELECTE S OCT 07 1988 D cu D			
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS		21. ABSTRACT SECURITY CLASSIFICATION	
22a. NAME OF RESPONSIBLE INDIVIDUAL DONALD S. BURKE		22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL

Detection of Flavivirus Antibodies in Human Serum by Epitope-Blocking Immunoassay

Donald S. Burke, Ananda Nisalak, and Mary Kay Gentry

Department of Virology, Armed Forces Research Institute of Medical Sciences, Rajvithi Road, Bangkok 10400, Thailand (D.S.B., A.N.); Department of Molecular Biology, Walter Reed Army Institute of Research, Washington, DC (M.K.G.)

Human flavivirus group-reactive, dengue complex-reactive, and encephalitis virus complex-reactive antibodies were detected using epitope-blocking immunoassays in which the binding of selected mouse monoclonal antibodies to flavivirus antigens was blocked by human serum. When late (> 6 months after illness) convalescent sera were tested, the epitope-blocking immunoassays were superior to the hemagglutination inhibition test and comparable to the plaque reduction neutralization for identifying subjects immune to dengue, to Japanese encephalitis, or both viruses.

Key words: dengue virus, Japanese encephalitis virus, serum antibodies, rapid viral diagnosis

INTRODUCTION

Viruses of the family flaviviridae (formerly Group B arboviruses) are major pathogens of humans capable of producing a variety of clinical syndromes, including fever, hemorrhagic fever, or encephalitis. In their seminal work on arbovirus taxonomy, Casals and Brown [1954] defined the Group B arboviruses as those viruses sharing common antigens in hemagglutination-inhibition tests. Antigenic cross-reactivity has not been determined for all flavivirus-encoded proteins, but it is clear that one or more major cross-reactive antigenic sites are on the virion surface. Individual viruses within the flavivirus family are differentiated on the basis of neutralization tests. Some complexes of individual viruses show significant cross-reactivity by neutralization tests. One such complex is comprised of the four serotypes of dengue viruses: dengue-1, dengue-2,

Accepted for publication February 17, 1987.

The views of the authors do not purport to reflect the positions of the U.S. Department of the Army or the U.S. Department of Defense.

Donald S. Burke is now at Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, DC 20307-5100.

Address reprint requests to the Academic Affairs Branch, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, DC 20307-5100.

dengue-3, and dengue-4. The flavivirus encephalitis viruses (Japanese encephalitis virus, St. Louis encephalitis, and others) comprise another complex [DeMadrid and Porterfield, 1974].

Although flaviviruses are important pathogens of humans, most infections with these viruses are associated with relatively mild illness, and many are entirely asymptomatic. In tropical regions of the world, the lifetime risk of experiencing at least one flavivirus infection may approach 100%. The most common flavivirus infection of humans on a global scale is dengue. The geographic zone of distribution of any given flavivirus may overlap that of other flaviviruses [Shope, 1980]. For example, in Thailand, Japanese encephalitis virus (JEV) and all four serotypes of dengue viruses are widely distributed and commonly infect humans. Immunity to one flavivirus does not confer immunity to other flaviviruses. During a lifetime in the tropics, many persons experience infections with several different flaviviruses.

A variety of assays are available for detection of antibodies to flaviviruses in human serum. The hemagglutination inhibition (HAI) test perfected by Clarke and Casals [1958] has been the standard method used in serologic surveys. Although all flaviviruses cross-react in the HAI systems, homologous reactions are typically stronger than heterologous reactions. Thus the sensitivity of the HAI test as a screening test for previous flavivirus exposure depends on whether the antigen selected is that of the predominant virus in the population under study. A common practice is to test sera with a battery of several different flavivirus antigens to avoid inadvertent selection of the incorrect antigens. Conversely, the presence of serum HAI antibodies does not prove prior infection with the test antigen virus; HAI antibodies only constitute evidence for past infection with an unspecified flavivirus.

We reasoned that a more precise, and perhaps simpler, approach for defining specific flavivirus exposure would be to examine sera for reactivity with specific epitopes. To do this we constructed a series of assays that measure the ability of a test serum to block the binding of an enzyme-labeled monoclonal antibody to its corresponding epitope on the viral antigen. We call this test the Defined Epitope Blocking Enzyme-Linked Immunosorbent Assay, or DEB-ELISA.

MATERIALS AND METHODS

Virus Strains

Prototype virus strains used were DEN-1 (Hawaii), DEN-2 (New Guinea C), DEN-3 (H87), DEN-4 (H241), and JEV (Nakayama). Virus that had been serially passed in suckling mouse brains was used in all assays.

Hemagglutination Inhibition and Neutralization Assays

Acetone extracted sera were tested for HAI antibodies with a microtiter adaptation of the method of Clarke and Casals [1958] using 4 to 8 hemagglutinin units per test. Neutralizing antibodies were measured by the plaque reduction method on LLC-MK2 cells using 50% reduction as the cut-off value [Russell et al, 1967].

Monoclonal Antibodies

Mouse monoclonal antibodies were prepared against each of the prototype viruses listed above using methods previously reported [Gentry et al, 1982; Henschel et al, 1982, 1983, 1985]. From a total of 165 clones secreting antibodies, 8 were selected for use in this

TABLE I. Titers of Selected Flavivirus Monoclonal Antibodies Assayed Against a Panel of Flavivirus Antigens

Clone	Immunogen	DEN-1	DEN-2	DEN-3	DEN-4	JEV	Others ^a
4G2	DEN-2 ^b	6.6 ^c	6.8	6.9	7.7	6.8	6.1
13G9	DEN-1	7.6	7.7	6.8	6.9	5.9	5.9
15H5	DEN-3	6.9	6.8	6.9	6.0	< ^d	<
13D4	DEN-1	5.7	5.7	5.9	6.7	<	<
11G5	JEV	<	<	<	<	7.9	<
1E6	JEV	<	<	<	<	7.8	<
8D2	JEV	<	<	<	<	6.9	<
2F1	JEV	<	<	<	<	6.2	<

*Three other flaviviruses native to Thailand were also tested: Tembusu, Wesselsbron, and Langat. The highest of these three titers is shown.

^bVirus used to immunize mice to prepare monoclonal antibodies.

^cLog 10 of titer that bound mouse brain antigen in solid phase immunoassay. $d \leq 3.0$

study. Properties of mouse ascitic fluid preparations derived from these clones are listed in Table I. Clones 4G2 and 13G9 are flaviruses group reactive, 15H5 and 13D4 are dengue complex reactive, and 11G5, 1E6, 8D2, and 2F1 are encephalitis virus complex reactive. Immunoglobulin purified from ascitic fluid by precipitation with ammonium sulfate was labeled with horseradish peroxidase by the two-step glutaraldehyde method [Engvall, 1980].

Defined Epitope-Blocking ELISA

1. All 96 wells of a polystyrene microtiter plate were sensitized with 100 μ l of a 1:1,000 of flavivirus hyperimmune antisera (human source) in alkaline carbonate buffer for 4 hours, then filled with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS-BSA) for 2 hours. The plates were washed with PBS containing 0.05% Tween-20.
2. Fifty hemagglutinating units of acetone-extracted suckling mouse brain-derived antigen in 25 microliters of 90% PBS-BSA and 10% acetone extracted normal mouse serum (NMS) were added to each well. The plate was stored refrigerated overnight, then washed.
3. Twenty-five microliters of the test serum dilution (in PBS-BSA) were added per well and incubated for 2 hours. Sera were not extracted with acetone before testing. Wells were not washed before the next step.
4. Twenty-five microliters of the enzyme-labeled conjugate diluted in 90% PBS-BSA/10% acetone extracted NMS were added per well and incubated an additional 1 hour. The dilution for each conjugate was selected as the highest dilution that still produced 80% of the maximum O.D. change. The plate was then exhaustively washed.
5. One hundred μ l of orthophenylene diamine substrate solution was added per well, and the color produced at 30 minutes was measured with a spectrophotometer.

Serial two-fold dilutions of sera were tested. Results were expressed as the titer that blocked 50% of the color obtained with a normal human nonimmune blocking serum.

Sera From Patients With Acute Flavivirus Infections

Acute, early convalescent (2 weeks), and late convalescent (6 months–2 years) serum specimens were obtained from patients with acute dengue infections (dengue fever

Special
A-1 21

or dengue hemorrhagic fever) or pyrexia of undetermined origin (PUO) at Bangkok Childrens Hospital through the courtesy of Dr. Suchitra Nimmannitya. A similar panel of serum specimens from patients with acute viral encephalitis was obtained during an epidemic of Japanese encephalitis in Kamphangphet, Thailand, through the courtesy of Dr. Thanom Laorkapongse. All cases of acute dengue were proven by virus isolation from blood, and all cases of JE were proven by detection of JEV IgM antibodies in cerebrospinal fluid [Burke et al, 1985]. Acute and 2-week convalescent sera were tested for HAI antibodies to all 4 dengue virus serotypes and to JEV. PUO cases were shown to lack flavivirus antibodies in both acute and convalescent sera. Acute flavivirus infections were classified as primary or secondary according to the magnitude of the HAI response; a 2-week convalescent HAI titer of $> 1:2,560$ to any of the 5 antigens tested was taken as evidence of an anamnestic, or secondary, antibody response.

Sera From a Prospective Longitudinal Study of Flavivirus Infections in a Region Where Both Dengue and JE Viruses Cocirculate

Thirty pairs of sera from children with serologic evidence of asymptomatic flavivirus infections were provided by Dr. Charles Hoke. The sera had been collected as part of a prospective study of Japanese encephalitis virus infections in Kamphangphet Province in which specimens had been collected from healthy children in May 1983, just before the Japanese encephalitis epidemic season (and just before the peak of dengue transmission as well), and again 6 months later. The 30 serum pairs selected for study were all free of flavivirus HAI antibodies (titers $< 1:10$) in the preseason specimen and had a titer in the postseason sample of $> 1:20$ to at least one of the 5 antigens tested.

Statistical Analysis of Results

Spearman rank correlation coefficients were calculated to determine correlations of HAI, PRNT, and DEB-ELISA test results.

RESULTS

Selection of Monoclonal Antibodies and Antigens

In a pilot experiment, early (2-week) and late (6 months to 2 years) convalescent sera from 2 JE-infected and from 4 dengue-infected patients (2 primary and 2 secondary infections) were tested for their ability to block the attachment of the monoclonal antibodies to various flavivirus antigens. Results are shown in Table II. The combination of 4G2/JEV antigen was chosen for further evaluation as the flavivirus-group assay, 15H5/DEN-3 as the dengue-complex assay, and 11G5/JEV as the encephalitis-complex assay. In these preliminary assays, early convalescent sera were found to frequently produce low-level cross-blocking in the heterologous assays, but late convalescent sera blocked only in the appropriate homologous assays. In subsequent experiments only late convalescent sera were evaluated.

Evaluation of the DEB-ELISA Using Sera From Patients With Well-Characterized Acute Illnesses

Late convalescent (6 months to 2 years) sera from 10 patients with primary dengue infections, five patients with primary JEV infections, ten patients with secondary dengue infections, ten patients with secondary JEV infections, and ten patients with fevers not attributable to flaviviruses were tested by HAI for antibodies to DEN-3 and JEV and by

TABLE II. Pilot Experiment: Blocking of Labeled Monoclonal Antibodies by Early and Late Convalescent Human Flavivirus Immune Sera

			Source of blocking human serum					
Labeled monoclonal	Label dilution	Antigen	Two-week convalescent			Six-month convalescent		
			Primary JEV infection (N = 2)	Primary dengue infection (N = 2)	Secondary dengue infection (N = 2)	Primary JEV infection (N = 2)	Primary dengue infection (N = 2)	Secondary dengue infection (N = 2)
Flavi group test constructs								
4G2	500	JEV	+++	+++	+++	+++	+++	+++
4G2	100	DEN-4	0*	+	+++	+	+++	+++
13G9	1,000	DEN-1	++*	+++	++*	++*	+++	+++
13G9	100	JEV	++*	++*	++*	++*	++*	++*
Den complex test constructs								
15H5	3,000	DEN-3	+	+	+++	0	+++	+++
13D4	200	DEN-4	0	0*	+	0	0*	0*
JE test constructs								
11G5	5,000	JEV	+	0	+	+++	0	0
1E6	1,000	JEV	++*	0	++*	+++	0	0
8D2	800	JEV	0*	0	0	++*	0	0
2F1	1,000	JEV	++*	0	+	++*	0	0

0 signifies no blocking with either sera (both $\geq 50\%$ of controls).

*signifies unsatisfactory results for construction of specific blocking assay.

+ signifies partial blocking with one serum (25 to 50% of control) and no blocking with the other.

++ signifies partial blocking (25 to 50% of control) with both sera.

+++ signifies strong blocking with both sera (both $< 25\%$ of controls).

DEB-ELISA for 4G2, 15H5, and 11G5 blocking antibodies. Results are shown in Table III. Late convalescent serum from all 35 flavivirus-infected patients (having HAI antibodies to dengue JEV or both) had readily detectable 4G2 DEB-ELISA antibodies (titer $\geq 1:10$), whereas none of ten sera unreactive in HAI tests had 4G2 DEB-ELISA antibodies. Sera from all 20 dengue-infected patients had positive 15H5 titers ($> 1:9$), whereas the corresponding 11G5 DEB-ELISA titers were negative, but for one with a titer of 1:7. Sera from the five primary JEV-infected patients all had 11G5 DEB-ELISA titers $> 1:6$, whereas the corresponding 15H5 DEB-ELISA titers were negative. All 15H5 and 11G5 DEB-ELISA titers were positive in the 10 sera of encephalitis patients with secondary seroresponse patterns. Spearman rank correlation coefficients between titers obtained using the five test methods on all 45 sera are shown in Table IV.

Evaluation of the DEB-ELISA Using Sera Collected During a Longitudinal Survey

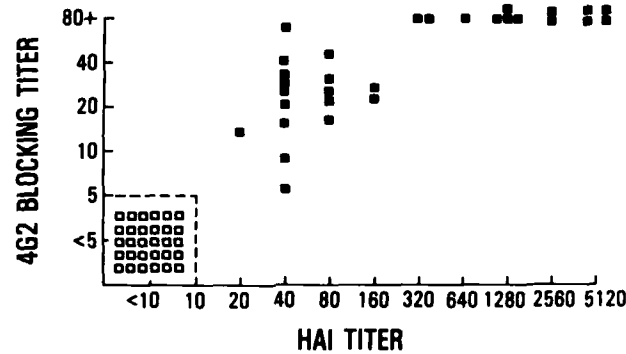
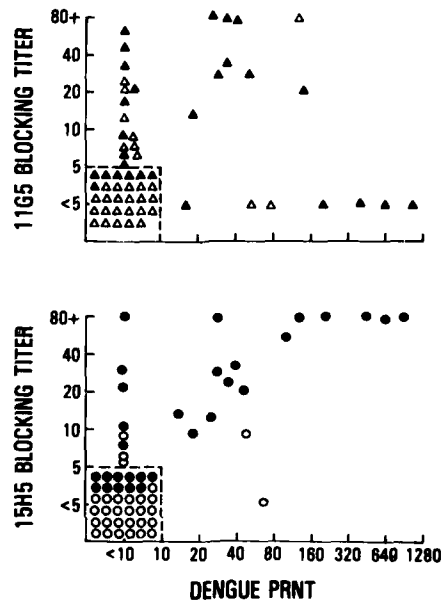
Serum pairs that showed a pre- to postseason conversion with the HAI test (from $< 1:10$ with all five antigens to $> 1:20$ with at least one antigen) were tested for blocking antibodies by 4G2, 15H5, and 11G5 DEB-ELISAs and for DEN-1, -2, -3, -4, and JEV PRNT antibodies. None of 30 HAI negative pre-season sera had 4G2 DEB-ELISA activity, whereas all 30 HAI positive post-season sera did. The relationship of the maximum HAI titer (JEV or dengue, whichever was greater) and 4G2 DEB-ELISA titers is shown in Figure 1. The magnitude of the 4G2 DEB-ELISA titer closely paralleled the

TABLE III. HAI and Epitope Blocking Activity in Late Convalescent Sera From Patients with Dengue, Encephalitis, or Pyrexia of Undetermined Origin (PUO): Comparison of Primary and Secondary Infections

Pat. no.	Disease	Acute HAI sero- response pattern	Titer in late convalescent sera				
			Den-3 HAI	JEV HAI	Flavivirus group (4G2) DEB	Dengue complex (15H5) DEB	Japanese encephalitis (11G5) DEB
1	DENGUE	PRIM	20	<10	140	42	<5
2	DENGUE	PRIM	40	20	179	110	<5
3	DENGUE	PRIM	20	10	23	9	<5
4	DENGUE	PRIM	20	<10	10	24	<5
5	DENGUE	PRIM	20	<10	32	80	<5
6	DENGUE	PRIM	40	<10	25	43	<5
7	DENGUE	PRIM	20	<10	44	26	<5
8	DENGUE	PRIM	80	20	44	151	<5
9	DENGUE	PRIM	20	20	41	23	<5
10	DENGUE	PRIM	10	<10	18	9	<5
11	ENCEPH	PRIM	<10	20	20	<5	24
12	ENCEPH	PRIM	<10	40	44	<5	45
13	ENCEPH	PRIM	20	640	1280	<5	866
14	ENCEPH	PRIM	<10	40	54	<5	52
15	ENCEPH	PRIM	<10	10	12	<5	6
16	DENGUE	SEC	80	80	448	452	<5
17	DENGUE	SEC	160	320	1280	273	<5
18	DENGUE	SEC	80	40	385	154	<5
19	DENGUE	SEC	320	640	1280	926	<5
20	DENGUE	SEC	80	40	644	344	<5
21	DENGUE	SEC	160	40	371	429	<5
22	DENGUE	SEC	40	40	267	647	<5
23	DENGUE	SEC	40	20	203	679	<5
24	DENGUE	SEC	80	640	1280	975	<5
25	DENGUE	SEC	80	160	686	953	7
26	ENCEPH	SEC	80	80	991	28	8
27	ENCEPH	SEC	160	320	1280	820	20
28	ENCEPH	SEC	40	80	552	174	41
29	ENCEPH	SEC	160	320	1280	172	9
30	ENCEPH	SEC	40	20	494	103	12
31	ENCEPH	SEC	20	80	1280	80	15
32	ENCEPH	SEC	20	40	1176	80	25
33	ENCEPH	SEC	160	320	1280	145	48
34	ENCEPH	SEC	320	320	1280	536	54
35	ENCEPH	SEC	40	40	717	689	22
36	PUO	NIL	<10	<10	<5	<5	<5
37	PUO	NIL	<10	<10	<5	<5	<5
38	PUO	NIL	<10	<10	<5	<5	<5
39	PUO	NIL	<10	<10	<5	<5	<5
40	PUO	NIL	<10	<10	<5	<5	<5
41	PUO	NIL	<10	<10	<5	<5	<5
42	PUO	NIL	<10	<10	<5	<5	<5
43	PUO	NIL	<10	<10	<5	<5	<5
44	PUO	NIL	<10	<10	<5	<5	<5
45	PUO	NIL	<10	<10	<5	<5	<5

TABLE IV. Spearman Rank Correlation Coefficients of Titers of 45 Late Convalescent Sera From Patients with Well-Documented Dengue Virus or Japanese Encephalitis Virus Infections

	4G2 DEB	DEN HAI	15H5 DEB	JEV HAI	11G5 DEB
4G2 DEB	1.000	.816	.749	.904	.492
DEN HAI		1.000	.870	.751	.085
15H5 DEB			1.000	.635	.024
JEV HAI				1.000	4.89


 Fig. 1. Scatter graph of HAI titers and 4G2 DEB-ELISA titers in 30 serum pairs. Preseason serum samples = $\square$ and postseason samples = $\blacksquare$. The Spearman rank correlation for postseason sera is .902.

 Fig. 2. Scatter graphs of dengue PRNT titers (maximum of DEN-1, -2, -3, and -4) versus 15H5 (lower panel) and 11G5 (upper panel) DEB-ELISA titers in 30 serum pairs. Pre- and postseason 15H5 DEB-ELISA titers = $\circ$ and $\bullet$; pre- and postseason 11G5 titers = $\triangle$ and $\blacktriangle$. The Spearman rank correlation coefficients between maximum dengue PRNT and 15H5 or 11G5 DEB-ELISA titers (for postseason sera) are .781 and .131, respectively.

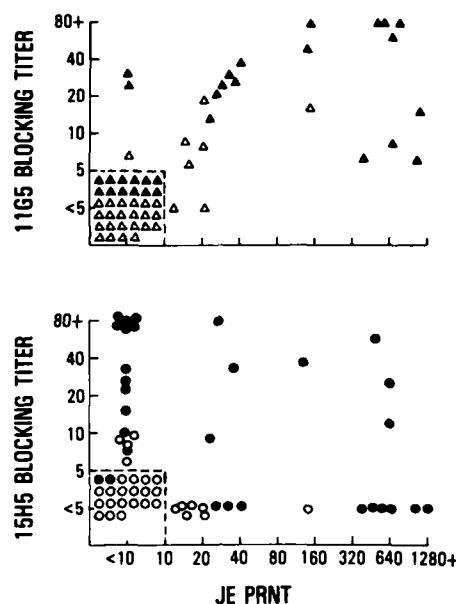


Fig. 3. Scatter graphs of JEV PRNT titers versus 15H5 (lower panel) and 11G5 (upper panel) DEB-ELISA titers in 30 serum pairs. Pre- and postseason 15H5 DEB-ELISA titers = $\circ$ and $\bullet$; pre- and postseason 11G5 titers = $\triangle$ and $\blacktriangle$. The Spearman rank correlation coefficients between JEV PRNT and 15H5 or 11G5 DEB-ELISA titers (for postseason sera) are $-.482$ and $.754$, respectively.

TABLE V. Spearman Rank Correlation Coefficients of DEN PRNT, 15H5/DEN-3 Blocking Titer, JE PRNT, and 11G5/JEV Blocking Titers in 30 Late Convalescent Serum Specimens

	DEN PRNT	15H5 DEB	JEV PRNT	11G5 DEB
DEN PRNT	1.000	.781	-.228	.131
15H5 DEB		1.000	-.482	-.149
JEV PRNT			1.000	.754

HAI titer. The relationships of the maximum (all four serotypes) dengue PRNT and JEV PRNT antibody titers with 15H5 and 11G5 DEB-ELISA titers are shown in Figures 2 and 3. Among postseason sera, 15H5 DEB-ELISA titers were closely correlated with dengue PRNTs ($R = .781$), and 11G5-DEB-ELISA titers were closely correlated with JEV PRNTs ($R = .754$). Other combinations showed either weak or negative correlations (Table V).

DISCUSSION

In this study we showed that it is possible to accurately determine the flavivirus immune status of individual humans by detection of serum antibodies directed against defined flavivirus epitopes. Results produced with our simple blocking immunoassays closely paralleled results obtained by more cumbersome conventional neutralization assays.

Several problems were overcome in the design and testing of the assays.

1. Not all mouse monoclonal antibodies with the desired specificity (flavivirus

group reactive or complex reactive) were equally suitable for use in blocking assays (see Table II). These differences may reflect differences in the immunodominance of different epitopes for humans and mice, inopportune spatial relationships of some epitopes with neighboring epitopes of broader cross-reactivity, or disadvantageous avidity properties of some of the monoclonal preparations.

2. In some instances selection of the antigen to be paired with the labeled monoclonal antibody was important. All four JEV immune sera tested in a pilot study failed to block 4G2 attachment to DEN-4 antigen, but blocked attachment of the same monoclonal antibody to JEV antigen quite well (Table II). Since the 4G2 antibody was raised against a dengue antigen (DEN-2), we interpret this observation to indicate that JEV immune sera failed to effectively compete with a highly avid homologous antibody/epitope pairing, but could do so effectively against a heterologous pairing.

3. False positive blocking was commonly encountered when early immune sera were tested (Table II). Although it is possible that a different array of epitopes is recognized by early and late immune sera, it is more likely that nonspecific steric blocking by large IgM molecules found in the early immune sera accounts for these false positive reactions.

These results suggest that flavivirus epitope-specific serologic assays can be constructed utilizing labeled synthetic polypeptide epitopes. Immunity can be assessed by direct measurement of binding between the serum and the labeled epitope probe. Peptides recognized by monoclonal antibodies, 4G2, 15H5, and 11G5 would appear to be logical candidates for use as diagnostic reagents.

REFERENCES

- Burke DS, Nisalak A, Ussery MA, Laorakpongse T, Chantavibul S (1985): Kinetics of IgM and IgG responses to Japanese encephalitis virus in human serum and cerebrospinal fluid. *Journal of Infectious Diseases* 151:1093-1099.
- Casals J, Brown LV (1954): Hemagglutination with arthropod-borne viruses. *Journal of Experimental Medicine* 99:429-449.
- Clarke DH, Casals J (1958): Techniques for hemagglutination and hemagglutination inhibition with arthropod-borne viruses. *American Journal of Tropical Medicine and Hygiene* 7:561-573.
- DeMadrid AT, Porterfield JS (1974): The flaviviruses (group B arboviruses): A cross neutralization study. *Journal of General Virology* 23:91-96.
- Engvall E (1980): Enzyme immunoassay: ELISA and EMIT. *Methods Enzymology* 70:431-432.
- Gentry MK, Henchal EA, McCown JM, Brandt WE, Dalrymple JM (1982): Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. *American Journal of Tropical Medicine and Hygiene* 31:548-555.
- Henchal EA, Gentry MK, McCown JM, Brandt WE (1982): Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. *American Journal of Tropical Medicine Hygiene* 31:830-836.
- Henchal EA, McCown JM, Burke DS, Seguin MC, Brandt WE (1985): Epitopic analysis of antigenic determinants on dengue-2 virions using monoclonal antibodies. *American Journal of Tropical Medicine and Hygiene* 34:162-169.
- Henchal EA, McCown JM, Seguin MC, Gentry MK, Brandt WE (1983): Rapid identification of dengue virus isolates by using monoclonal antibodies in an indirect immunofluorescence assay. *American Journal of Tropical Medicine and Hygiene* 32:164-169.
- Russell PK, Nisalak A, Sukhavachana P, Vivona S (1967): A plaque reduction test for dengue virus neutralizing antibodies. *Journal of Immunology* 99:285-290.
- Shope RE (1980): Medical significance of togaviruses: An overview of diseases caused by togaviruses in man and in domestic and wild vertebrate animals. In Schlesinger RW (ed): "The Togaviruses: Biology, Structure, and Replication." New York: Academic Press, pp 47-77.