

Group B Arbovirus Serology: Search for Humoral Specificity

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DESCRIPTION: It is possible to identify a primary group B arbovirus infection with considerable reliability by the standard arbovirus serological techniques of hemagglutination-inhibition (HI), complement fixation (CF), or plaque reduction neutralization (PRNT). However, in regions where multiple group B arboviruses are endemic, determination of the virus that produced infection is more difficult. The difficulty is caused by the broad serological response engendered by more than one group B arbovirus infection. Thus, repeated infections by dengue, Japanese encephalitis, or both viruses in the same individual cause that individual to form antibodies to JEV, all 4 dengue serotypes and related group B viruses, such as Tembusu. The antibody titers produced against the non-offending viruses are often as high as the titers produced against the actual infecting agent, and elevated titers persist for years. We have encountered many such serological cross-reactions during routine HI testing of serums obtained from human and animal residents of Chiangmai Valley, where dengue, JE, and Tembusu viruses circulate. The specificity—or lack of specificity—of the three standard serological tests was reexamined in groups of subjects with secondary group B infections. Certain serums broadly reactive to group B antigens in HI tests were selected for comparative HI, CF and PRN tests with the expectation that one, or a combination of serological tests, would identify a specific virus infection. The results of these serological investigations are reported below.

Results. Serological response of Thai children to Japanese encephalitis virus infection.

Table 1 shows the serological response to infection in three Thai children with encephalitis. One patient, JE-L-3, had a primary JEV infection and is included for purposes of comparison with secondary antibody titer patterns. Primary and secondary HI antibody responses to JEV infection are defined in Table 1a. As shown in Table 1, any one of the three serological tests provided a specific diagnosis of a recent primary JEV infection. However, none of the three serological tests could identify the specific infecting agent in the 2 patients with a recent secondary group B arbovirus infection, presumably recent JEV infections on clinical and epidemiological grounds. Specifically, there is a 4-fold or greater antibody titer rise to JEV and to one or more dengue antigens in each of the three tests, and the high, elevated titers against JEV and dengue antigens do not permit a conclusion as to the causative agent.

Serological response and viremia in dengue-sensitized gibbons challenged with JEV.

During 1968-1969 a number of splenectomized gibbons lacking pre-existing group B arbovirus antibody were sequentially infected with different dengue serotypes. The gibbons were housed in mosquito-proof rooms in the Department of Veterinary Medicine prior to and during the current study. Approximately 1 year after their last dengue inoculation, 8 gibbons were inoculated subcutaneously with 1×10^5 PFU of JEV. Blood was drawn for antibody titrations at weekly intervals for 3 weeks. Serum for virus isolation was obtained 3, 6, and 8 days post-inoculation. Table 2 records the prior dengue exposure of the gibbons, the presence or absence of JE viremia, and the antibody response after JEV challenge. The gibbons were not ill after JEV challenge.

JE viremia was detected in 5 of 8 animals, and of those 5, 3 gibbons had pre-existing dengue neutralizing antibody at a titer of 1:40 or greater. One viremic gibbon had a pre-existing JEV neutralizing antibody titer of 1:10. Thus the presence of group B arbovirus neutralizing antibody prior to challenge was not correlated with protection against viremia, at least at antibody levels of 1:10 for JE and 1:40-1:160 for dengue. It is possible, of course, that neutralizing antibody could protect against viremia if the challenge dose of virus were lower than 10^5 PFU.

The serological responses of the 8 gibbons were remarkably uniform; all showed diagnostic HI, CF, and PRN antibody titer rises to JEV and to one or more of the four dengue serotypes. The highest JEV titers were no more than 4 times the highest dengue HI titer, and equal to or less than the highest dengue CF and PRN titers. Thus the CF and PRN tests could not identify the most recent group B infection (JEV) in these dengue-sensitized gibbons.

Serologic response in yellow-fever vaccinated soldiers to Japanese encephalitis virus infection.

For presentation of these serological results and their discussion, refer to Table 2 in another section of this Annual Report entitled, "Japanese Encephalitis among United States Military Personnel in Vietnam". In summary, the PRNT merely confirmed the HI and CF tests and was unable to eliminate antibody cross-reactions to dengue in these Americans with prior group B sensitization.

Serological response to group B arbovirus antigens in children with dengue hemorrhagic fever.

This study was designed to test the thesis that repeated dengue infections would not produce a rise in JEV antibody. Seven Bangkok children with dengue hemorrhagic fever were selected because of their residence in Bangkok, a city presumed to be free of Japanese encephalitis. The serologic results, listed in Table 3, show that HI, CF, and PRNT antibodies are produced to JEV after second dengue infections. However, the JEV neutralizing antibody titers in 6 of 7 convalescent serums are lower than dengue 1-4 PRNT titers by a factor of 1/4-1/20 or more. This PRNT serological pattern seems to be characteristic of repeated dengue infections and was not observed in other infection sequences such as dengue-JEV (gibbons), yellow fever-JEV (American Soldiers), or dengue (?) -JEV and JEV (?) -JEV (encephalitis in Chiangmai). The combination of a low rising JEV titer with high rising dengue 1-4 titers, confirmed in the three standard serologic tests, may indicate a recent dengue infection in patients previously sensitized to dengue but not to JEV.

Group B arbovirus antibody titers in healthy Thais.

The purpose of this limited serological study was to determine if HI, CF, or PRN tests can identify the most recent infecting agent in subclinical cases of second group B infections. We examined the antibody patterns in several healthy Chiangmai villagers who gave no history of recent illness. The serological patterns of one representative subject listed in Table 4 show that antibody titers are nearly equally elevated and fixed to JEV and to dengue 1-4 antigens. Therefore routine serological tests of subclinical secondary group B infections, as in clinically apparent infections, do not provide suitable serological specificity.

The sum total of the serological data in humans and gibbons reported above indicates that antibody reacting equally well with all group B antigens in conventional serological tests prevents reliable assessment of the force of either JEV or dengue virus infections in an environment where both viruses circulate.

Table 1. Serological Results In Three Chiangmai Children with Encephalitis

Patient No.	Serum days after onset	Antibody titre vs				
		JEV	D4	D3	D2	D1
JE-L-3 (primary infection)				<u>HI</u>		
	3	0 ^(a)	0	0	0	0
	7	320	40	0	0	0
				<u>CF</u>		
	3	0 ^(b)	0	0	0	0
	7	8	0	0	0	0
				<u>PRNT^(c)</u>		
	3	<10	<20	<20	<20	<20
	7	160	<40	<40	<40	<40
JE-C-2 (secondary infection)				<u>HI</u>		
	3	320	320	320	160	320
	13	2560	1280	1280	640	640
				<u>CF</u>		
	3	16	32	32	64	32
	13	256	256	128	256	256
				<u>PRNT</u>		
	3	400	40	640	450	400
	13	1280	100	2560	2560	2560
JE-M-2 (secondary infection)				<u>HI</u>		
	4	320	640	320	160	320
	13	2560	1280	1280	320	640
				<u>CF</u>		
	4	16	128	128	64	32
	13	128	512	512	512	256
				<u>PRNT</u>		
	4	<40	40	300	620	550
	13	320	100	640	2200	2000

a) 0 for HI = <20 (reciprocal of the serum dilution vs. 8 units of indicated antigen)

b) 0 for CF = <4 (reciprocal of the serum dilution vs. 4 units of antigen)

c) reciprocal of 50% plaque reduction titers

Table 1a. Definition of Primary and Secondary HI Antibody Responses to JEV Infection.

<u>Response</u>	Serum HI antibody titer	
	Acute	Convalescent
primary	dengue 1-4 \leq 1:20	JE \geq 4 times dengue 1-4
secondary	dengue 1-4 \geq 1:40	JE \leq 2 times dengue 1-4

Table 2. Serological Response and Viremia in Dengue--Sensitized Gibbons Challenged with JEV (a).

Gibbon Past Dengue Infections	No. days after JEV (a)	Viremia JEV	HI				CF				PRNT								
			D ₁	D ₂	D ₃	D ₄	D ₁	D ₂	D ₃	D ₄	D ₁	D ₂	D ₃	D ₄	JEV				
			D ₁	D ₂	D ₃	D ₄	D ₁	D ₂	D ₃	D ₄	D ₁	D ₂	D ₃	D ₄	JEV				
S61 (b) (D1,4,2)	0	neg.	20	20	20	40	<20	4	8	4	4	4	4	<4	40	<40	40	<10	
	7		40	40	40	40	20	8	16	8	8	8	8	<8	160	<40	160	<40	
	14		80	80	80	80	160	16	32	32	16	16	32	32	160	<40	500	160	
	21		160	160	320	160	640	64	64	64	64	64	32	32	400	2560	<160	500	1,000
S71 (D4,2,3)	0	neg.	<20	20	20	20	<20	4	4	4	4	8	8	<4	<40	160	<10	80	<10
	7		20	20	40	20	40	8	8	8	16	16	16	<8	<40	>160	<40	160	<40
	14		40	80	80	40	140	16	32	16	32	32	16	16	160	160	<40	640	40
	21		80	160	160	80	320	16	32	32	32	32	16	16	<160	640	160	160	640
S51 (D2,2)	0	pos.	<20	<20	<20	<20	<20	<4	<4	<4	<4	<4	<4	<4	<40	<40	<40	<40	<10
	7	days 3-6	<20	<20	<20	<20	<20	<4	<4	<4	<4	<4	<4	<4	<40	40	<40	<40	<40
	14		160	320	640	160	640	16	32	64	64	64	64	64	160	640	40	180	800
	21		320	320	640	320	1280	32	32	64	64	32	04	04	160	600	<160	640	800
S94 (D3,4,2)	0	pos.	<20	40	<20	<20	<20	<4	<4	<4	<4	<4	<4	<4	<40	<40	<40	<40	<10
	7	day 3	40	40	80	20	40	<8	<8	<8	16	16	16	<8	<40	40	<40	110	<40
	14		160	160	1280	160	1280	8	16	32	64	64	32	32	160	640	40	160	160
	21		160	160	320	160	1280	8	16	32	64	64	32	32	160	600	<160	1000	2000

(a) JEV strain 40783; SM3: Isolated from Human Brain, Chiengmai, Thailand 1969

(b) Dengue serotypes 1,4,2, serially inoculated 1 to 3 years before JEV.

Table 2. (Continued)

Gibbon Past Dangue Infections	No. days after JEV (a)	Viremia JEV				HI				CF				PRNT			
		D ₁	D ₂	D ₃	D ₄	JEV	D ₁	D ₂	D ₃	D ₄	JEV	D ₁	D ₂	D ₃	D ₄	JEV	
S36 (D2,3,4)	0	20	40	40	20	20	4	4	4	4	4	4	4	4	4	4	4
	7	20	40	40	20	40	4	4	4	8	4	4	4	4	4	4	4
	14	160	160	640	160	640	32	64	32	128	32	64	32	128	32	64	32
	21	160	320	640	160	640	32	32	32	128	32	32	32	128	32	32	128
S81 (D2,3,4)	0	40	40	80	20	20	4	4	4	4	4	4	4	4	4	4	4
	7	40	40	80	20	40	8	8	8	8	8	8	8	8	8	8	8
	14	40	80	320	40	160	8	8	8	16	16	16	16	16	16	16	16
	21	80	80	1280	80	320	16	16	32	32	32	32	32	32	32	32	32
S92 (D3,4,1)	0	20	20	20	20	20	4	4	4	4	4	4	4	4	4	4	4
	7	20	40	80	20	80	8	8	8	8	8	8	8	8	8	8	8
	14	320	320	640	320	1280	16	32	64	128	64	128	64	128	64	128	64
	21	320	320	640	320	2560	32	64	256	512	128	64	256	512	128	64	256
S70 (D2,3,4)	0	20	40	80	40	40	16	8	4	8	8	8	8	8	8	8	8
	7	20	40	40	20	20	8	16	8	16	8	16	8	16	8	16	8
	14	40	80	160	80	160	32	64	32	64	32	64	32	64	32	64	32
	21	160	160	320	160	640	32	128	32	128	32	128	32	128	32	128	32

Table 3. Serological Response to Group B Arbovirus Antigens in Children with Dengue Hemorrhagic Fever

Pt. No.	Date	HI				CF				PRNT						
		D ₁	D ₂	D ₃	D ₄	JEV	D ₁	D ₂	D ₃	D ₄	JEV	D ₁	D ₂	D ₃	D ₄	JEV
CH 0086	2 June 69	640	320	320	320	80	128	64	128	128	32	700	400	50	550	<10
	19 " "	10240	2560	> 20480	1280	1280	1024	512	2048	4096	512	> 10240	8000	5120	> 10240	270
CH 0094	5 June 69	80	160	320	160	40	32	32	32	64	8	160	160	640	22	<10
	19 " "	2560	2560	> 20480	2560	640	2048	1024	2048	1024	512	2000	5120	6000	1600	<40
CH 0072	26 May 69	160	640	1280	1280	320						160	640	<40	250	<40
	13 June "	2560	10240	> 20480	20480	20480		not done				4000	10240	1100	7000	160
CH 0081	29 May 69	160	160	2560	320	80						> 160	160	> 640	250	<40
	13 June "	2560	640	20480	20480	1280		not done				> 2560	> 2560	> 2560	800	40
CH 0066	21 May 69	80	160	160	80	40	64	32	64	32	16	160	> 160	> 160	70	<10
	6 June "	20480	2560	1280	640	1280	1024	512	2048	4096	1024	1024	1024	> 2560	> 2560	70
CH 0019	23 April 69	640	1280	5120	1280	640	512	256	512	512	256	ND	ND	640	1300	160
	12 May "	5120	10240	20480	20480	10240	2048	4096	2048	4096	2048	> 10240	> 10240	2560	2560	3000
CH 0015	21 April 69			not done								ND	ND	200	700	40
	7 May "	> 20480	20480	1024	2560	5120	1024	1024	1024	1024	256	> 10240	> 10240	640	1900	160

Table 4. Antibody response to group B antigens in a healthy resident of Chiangmai

Donor No.	Date Serum Collected	Antibody Titers against				
		JEV	D4	D3	D2	D1
JE-S-2	3 June	1280	1280	<u>HI</u>	320	640
				1280		
	17 June	1280	1280	<u>CF</u>	16	16
				16		
				<u>PRNT</u>	400	270
				100		
					800	320
			160	200	160	

The preceding 5 serological investigations lead to the conclusion that no one standard serological test, employed alone or in concert with two other standard tests, reliably identifies recent or past infecting viral strains in individuals with repeated group B arbovirus infections. The one exception may be found in repeated dengue infections, where results of the three tests taken together may distinguish between dengue and JEV, but not between dengue serotypes; but even this conclusion will require more substantiation.

Several serological methods have been employed to provide increased specificity in group B infections, such as arboviral kinetic neutralization tests, kinetic HI tests, and antigen absorption HI tests. However these methods are technically delicate and tedious, and are not readily adaptable for routine use in the diagnostic or field lab where large numbers of serum specimens must be quickly processed. We therefore have sought other methods, which are described below.

The Effect of Serum Accessory Factor (fresh human serum) on JEV & Dengue Virus Neutralizing Antibody. Serum accessory factor (AF) is a heat labile component of freshly drawn animal serum. When added to immune serum of the homologous species, AF will increase the neutralizing antibody titers against arboviruses of groups A & B. The mechanism of its action is unknown.

The purpose of this study was to test whether human serum accessory factor can increase the specificity of neutralizing antibody activity in serums from patients showing secondary group B arbovirus infections. Fresh frozen human serum, obtained from two American volunteers having no history of arbovirus exposure, was kindly supplied by COL Dan Crozier, US Army Institute of Infectious Diseases, and COL P.K. Russell, WRAIR. One of the two serums which had no neutralizing activity against Dengue 1-4 and JEV at serum dilutions of 1:2 and 1:10 was used as a source of AF for these experiments. Indices of serum heat-labile complement activity were in the normal range (hemolytic complement titer of 1:8; Beta 1c/1a concentration of 145 mg%) thereby providing evidence that the serum was not exposed to excess heat and possible inactivation of AF before it was frozen. The serum, henceforth called AF, was stored frozen at -90°C , and small aliquots were thawed immediately before use. Thawed but unused AF was discarded. One part of AF was mixed with one part virus diluent. The resulting virus-AF mixture was in turn mixed one to one with dilutions of inactivated (56°C , 30 min) immune serum. Thus the final dilution of AF was 1:4. The virus-immune serum mixture with and without AF was incubated for 30 seconds and 30 minutes. The reaction mixture was then placed on ice immediately before it was layered on MK2 cell monolayers. Virus neutralization for 30 seconds yielded the same antibody titers as neutralization for 30 minutes. In recent unfinished experiments, the virus-serum mixtures were incubated for 30 seconds, 90 seconds, and 30 minutes, and the immune reactions stopped by diluting the reaction mixture 20-fold rather than chilling; a more complete inhibition of virus neutralization should theoretically be achieved with dilution. Table 5 illustrates the effect of AF on JEV and dengue neutralizing antibody titers in the serum of patients with presumed JE. One patient had a secondary group B arbovirus infection by HI test (JE-C-2) and the other patient had a primary JEV infection (JE-P-22); accessory factor enhanced the neutralizing titer of acute and convalescent serums 2 to 20 fold. The higher neutralizing titers with AF was not specific for JEV, as anti-dengue titers were enhanced equally well. AF added to inactivated serum containing no detectable antibody to dengue (JE-P-22), had no enhancing effect. It is likely, therefore, that AF acts directly on antibody or antigen-antibody complexes rather than directly on the virus.

In order to confirm the potentiation of AF on JEV and dengue neutralizing antibody, fresh human serum was added to acute and convalescent serum obtained from a patient hospitalized with dengue hemorrhagic fever (pt. CH 0009-69, Table 1). The results shown in Table 5 indicate that AF increased both anti-JEV and anti-Dengue 4 titers by 10 to 25 times. We conclude that AF is unable to selectively enhance antibody titers against the most recent infecting agent—be it JEV or dengue virus—in patients who have experienced previous sensitization to group B arbovirus. The use of human accessory factor does not provide a solution to the diagnostic problem of serological cross-reactions in group B arbovirus infections.

Table 5.

The Effect of Serum Accessory Factor on Neutralizing Antibody Titers in the Serum of Patients with JEV and Dengue Virus Infections.

Patient No. and Serum	Serological Test	AF added	Antibody titers against				
			JEV	D4	D3	D2	D1
(1) JE-C-2 (acute serum)	HI	no	320	320	320	160	320
	PRNT	no	10	10	70	10	<160
	PRNT	yes	150	200	120	90	450
(convalescent serum)	HI	no	2560	1280	1280	640	640
	PRNT	no	160	<40	250	80	800
	PRNT	yes	2560	1500	700	500	2000
(2) JE-P-22 (acute serum)	HI	no	<20	<20	<20	<20	<20
	PRNT	no	40	<10	<10	<10	<10
	PRNT	yes	>160	<10	<10	<10	<10
(convalescent serum)	HI	no	640	80	40	20	20
	PRNT	no	160	<10	<10	<10	<10
	PRNT	yes	3200	<40	<10	10	10
(3) CH-0009-69 (acute serum)	HI	no	5120	20480			
	PRNT	no	270	1900	N.D.	N.D.	N.D.
	PRNT	yes	>10,240	25,000			
(convalescent serum)	HI	no	10240	10240			
	PRNT	no	120	640	N.D.	N.D.	N.D.
	PRNT	yes	7000	6000			

- (1) Thai child with Japanese encephalitis = secondary group B arbovirus infection by HI test.
 (2) Thai child with Japanese encephalitis = primary JEV infection by HI test
 (3) Thai child with dengue hemorrhagic fever = secondary group B arbovirus infection by HI test
 N.D. = not done

Serum IgM Antibody Specificity in Group B Arbovirus Infections.

Rabbit serum IgM has been shown to carry greater antibody specificity than serum IgG in HI tests run against closely related group B arbovirus antigens (E.G. Westaway, *Nature* 219, 78, 1968) COL P. K. Russell has reported that following a dengue virus infection in a group B arbovirus sensitized man (prior yellow fever vaccination) serum IgM rather than IgG carried the higher degree of antibody specificity for dengue virus. (WRAIR, Annual Progress Report, 1970). We therefore sought to expand these limited observations and test for the presence and specificity of IgM contained in animal and human sera which showed cross reactivity to JEV and dengue antigens in the standard serological tests.

Treatment of whole serum with 2-mercapto-ethanol (2-ME)

We first tested for the presence of serum IgM antibody by treating whole serum with 2-ME. If the HI antibody titre after treatment was reduced to, or below one-fourth the titre before treatment, the serum was judged to contain 2-ME sensitive (IgM) antibody. The method of 2-ME treatment was as follows. One part of a 1:10 dilution of 2-ME in borate saline (pH 9.0) was added to 9 parts of red cell-adsorbed, acetone-treated serum. The mixture was incubated at 37°C for 30 minutes and then placed at 4°C for 30 minutes. 2-ME treated and untreated serum were then diluted simultaneously for the HI test.

We tested sera obtained from 4 sentinel pigs stationed in Chiengmai, from 2 gibbons inoculated with dengue and JEV in the laboratory, and from 8 Thai and 12 American patients hospitalized with Japanese encephalitis in Chiengmai and Vietnam.

The results are shown in Tables 6 and 7.

Table 6.
2-ME Sensitivity of Serum Obtained from Sentinel Pigs
Showing Recent* HI Antibody Conversion.

Pig No.	Serum HI antibody titers			
	JE		D2	
	Before 2-ME	After 2-ME	Before 2-ME	After 2-ME
1	320	640	80	80
2	320	320	40	40
3	320	320	20	<20
4	160	40	20	<20

* HI antibody titre 30 days previously was <1:20 against JEV & D2

Only 1 of 4 sentinel pigs (pig #4) displayed detectable 2-ME sensitive JEV antibody in their whole serum, even though all 4 pigs suffered a recent group B infection. No 2-ME sensitive antibody was detected that reacted against dengue antigens 1-4 (representative results against one dengue serotype are listed in Table 6). The absence of a detectable IgM response to JEV in 3 of 4 pigs may be the result of an insensitive test procedure (serum IgM fractionation may offer more sensitivity), a transient anti-JEV IgM response missed in the one blood sample tested, or infection with a group B virus other than JEV. These 3 possibilities are currently being investigated.

Two gibbons were tested for IgM antibody 7, 14, 21, and 28 days after an immunizing dose (1×10^5 PFU) of JEV. Neither gibbon contained detectable IgM activity in their serums (Table 7). For a detailed history of dengue and JE virus infections and antibody response in these animals, refer to Table 2 (gibbons S-51 and S-92).

Acute and convalescent serums were tested from 12 American soldiers with encephalitis. The serums were obtained 2 to 31 days after onset of illness. HI, CF and PRN test results showed ≥ 4 fold antibody titer rises against JEV and one or more dengue serotypes indicating they had been infected previously by a group B virus. The 12 soldiers had in fact been immunized with yellow fever vaccine before their illness and they were exposed to dengue virus in Vietnam. The sera from only 2 donors contained 2-ME sensitive antibody directed against JEV but not dengue 1-4 (Table 7).

The results of serum analysis in 8 Thai children hospitalized with Japanese encephalitis were similar to results found in American soldiers. Of 8 paired serums tested, only 4 donors had evidence of IgM antibody reactive against JEV in one or both serum pairs. It is of interest that IgM reactive against JEV was detected in the serums of 6 of 7 Thai & American patients with primary HI antibody patterns to JEV, but in none of 13 donors with secondary HI antibody patterns.

These results in man indicate that IgM antibody does appear in the majority of patients with a primary immune response to recent JEV infection, and that the antibody is specific for JEV. However, using 2ME treatment of serum we are unable to demonstrate IgM in the serums of patients that show a secondary-type antibody response to a recent JEV infection. Since a primary immune response to JEV can be readily distinguished by routine CF, PRN, or HI tests, while secondary responses to JEV cannot, 2ME treatment of whole serum offers no real diagnostic advantage.

Table 7. 2-ME Sensitivity of Gibbon and Human Serum Obtained After Recent JEV Infections.

Donor	No. donors	No. Serum samples (a) treated per donor	No. donors positive (b)	Percent donors positive
gibbon ^(c)	2	4	0	0
American soldiers ^(d)	12	2	2	17
Thai children ^(e)	8	2	4	50

- (a) Acute and convalescent bloods drawn 7-28 days after JE inoculation (gibbon) and 2-31 days after illness (human).
- (b) Positive results defined as \geq 4-fold HI titer fall to JEV after 2ME treatment of acute and/or convalescent serum, and no HI titer drop (\leq 2) against dengue 1-4.
- (c) Gibbons inoculated 2 to 3 times with dengue virus 1 to 3 years before JEV inoculation.
- (d) Hospitalized in Vietnam with Japanese encephalitis in 1970.
- (e) Hospitalized in Chiangmai with Japanese encephalitis in 1970.

Isolation of serum IgM antibody by sucrose density gradient centrifugation.

Although 2-ME treatment of whole serum did not permit detection of immunoreactive IgM in the serum of patients with secondary group B arbovirus infections, we had reason to suspect that IgM did indeed exist in such serums from the previous work of Westaway and Russell. IgG exists in high titer in most serums following secondary group B infections and is resistant to 2-ME degradation. We reasoned that IgM antibody activity in whole serum may be masked by serum IgG activity; we therefore elected to isolate IgM from IgG by sucrose density gradient centrifugation (S-DC) of serum specimens. This technique provides several advantages for immunoglobulin separation not offered by ion exchange or molecular sieve chromatography; these advantages can be seen in Table 8, where a comparison of S-DC and chromatography are summarized.

The fractionation of serum by S-DC followed standard methodology. Briefly, 0.125 ml of inactivated serum was diluted with normal saline to 0.25 ml, adsorbed with goose erythrocytes, and the diluted serum layered on a 10% to 40% sucrose gradient (5.5 ml total volume). Following centrifugation at 35,000 RPM for 18 hours, 12 fractions of the gradient were collected dropwise through a pin-hole drilled in the bottom of the centrifuge tube. Fractions 1 thru 7, which contained IgM, were collected in 0.3 ml aliquots, while fractions 8-12, which contained the bulk of the IgG, were collected in 0.5 ml volumes. Each fraction was divided into two aliquots. One aliquot was treated with 2-ME (.135 ml sucrose fraction plus 0.15 ml of 0.2 molar 2-ME) for 30 minutes at 37°C and then at 40°C; the second aliquot was treated with 0.15 ml of buffer similarly. An extended incubation time of 60 minutes with 2-ME did not enhance IgM degradation. The untreated (control) and treated aliquots were then diluted for HI titration against 8-16 units of JE and dengue 1-4 antigens. IgM and IgG concentrations in the untreated fractions of selected serums were determined by radial immunodiffusion in agar using Hyland Lab "Immunoplates". The presence of IgM immunospecific HI antibody activity was shown by its characteristic position in the sucrose gradient, by determination of the IgM content of each fraction, and by its susceptibility to reduction by 2-ME.

Representative results using the S-DC fractionation procedure are listed in Tables 9 and 10, where convalescent and acute phase serum samples were assayed from a Thai child ill with suspected Japanese encephalitis. The HI test results on the whole serum indicated the child suffered from a secondary group B arbovirus infection, virus-type unspecified (see "whole serum", Table 9 & 10). Fractionation of the 15 day convalescent serum (Table 9) revealed that all of the IgM detectable by immunodiffusion was concentrated into fractions 3 thru 6, whereas only 17% of the total IgG was located in these 4 fractions. Immunospecific IgM is considered present when the HI titer against one of 5 different antigens (JEV, D1-4) falls after 2-ME treatment. The fall is considered significant when 2-ME treatment reduces the HI titer to 1/8 of the control titer in any one fraction, or to 1/4 of the control in two or more fractions. In Table 9 note that 2-ME sensitive HI antibody, reactive in low titer against JEV but not reactive against dengue antigens 1-4, is located in fractions 1-6. Note further the presence of high titer of antibody to JEV & dengue 1-4 in fractions 7-12 which is resistant to 2-ME degradation. The antibody remaining after 2-ME treatment is undoubtedly IgG, present in small concentrations in fractions 2-6, and in large concentrations in fractions 7-12 (Table 9).

No immunospecific IgM antibody was detected in the acute phase serum obtained 2 days after onset of illness despite the presence of IgM in this serum (fractions 4 & 5, Table 10).

It is of interest that 2-ME sensitive antibody activity was found in fractions 1 and 2 of the convalescent serum (Table 9). These 2 fractions contained no detectable IgM by radial immunodiffusion assay. This finding suggests that if IgM immunospecific for JEV or D1-4 is present, it can be detected more readily by the HI test, which measures functional antibody activity, than by the immunodiffusion assay. Since the immunodiffusion assay cannot detect an IgM concentration less than 2.75-5.5 mg%, the minimum concentration of IgM antibody capable of giving a positive HI test must be less than 2.75 mg%.

From the results presented in Table 9 and 10 we conclude that the S-DC procedure partially isolates IgM from IgG and thereby permits measurement of IgM antibody titer. We further conclude that IgM is produced in a secondary group B arbovirus infection and is detectable at 15 days but not 2 days after onset of illness. Finally IgM antibody reacts against the infecting virus in low titers, while IgG antibody reacts with the antigen or antigens common to JEV and dengue 1-4 in high titers. The presence of immunoreactive IgM against JEV in this encephalitis patient from Chiangmai provides strong additional serological confirmation of a recent JEV infection.

The virus-specificity of IgG poor, IgM rich, S-DC fractions was tested further in groups of patients with different secondary group B infections. We first studied acute and convalescent sera from 7 Chiangmai children hospitalized with acute encephalitis during the 1970 JEV epidemic. Six of the 7 showed rising or fixed HI titers to JEV and to one or more dengue serotypes. The CF antibody titers determined in several patients were not specific for JEV. One of 7 patients had negative CF titers but fixed, elevated HI antibody titers reacting monospecifically against JEV. The serum of all 7 patients contained 2-ME sensitive IgM antibody reactive against JEV but not against dengue antigens. The finding of IgM immunospecific for JEV was confirmatory of a recent JEV infection in these patients. The one child with the fixed, elevated anti-JEV titers was of particular epidemiological interest. This patient was initially hospitalized with encephalitis in Chiangmai in April 1970. Because she lacked rising titers to JEV in conventional serological tests, she was initially overlooked as the index case of the 1970 epidemic. The positive IgM serology suggested she indeed had suffered an acute JEV infection, and that the acute serum was drawn too late to detect a titer rise by routine serological methods.

We next studied the convalescent serums from 22 American soldiers hospitalized in Vietnam with suspected Japanese encephalitis. The clinical, epidemiological, and serological background of this group of patients is discussed in another section of this annual report. (See "Japanese encephalitis among United States military personnel in Vietnam"). Twenty-two patients showed rising or high fixed HI (≥ 640), or CF (≥ 32) antibody titers to JEV and to one or more dengue serotypes; dengue titers equalled or exceeded the JEV titers in eleven of these patients. Only two patients showed clear monospecific HI antibody titer rises to JEV. Twenty-one of the 22 patients had IgM in their serums reactive against JE antigen but not against dengue 1-4 antigen, thus indicating a recent infection with JE virus.

Five adults with acute psychosis, admitted to Suan Prung Psychiatric Hospital in Chiangmai during the 1970 JE epidemic, were examined for IgM antibody. Four of the 5 patients had fixed, elevated titers monospecific for JEV, while one patient had fixed titers elevated against JEV and dengue. Two of the 5 patients had IgM reactive against JE antigen; however, one serum was drawn 75 days after the onset of psychosis and more than one month after hospital discharge, so the patient may have experienced a subclinical JE infection during hospitalization or after his discharge. The second patient's serum was drawn 7 days after the onset of psychosis, thereby providing evidence for temporal association between JEV infection and psychosis. Because there have been no previous reports of acute JEV infections presenting as an acute psychosis, psychotic and control patients will be re-examined for IgM antibody to JEV during the 1971 encephalitis season.

Three healthy Chiangmai villagers were examined for IgM antibody. Each had elevated fixed titers to dengue and JEV. No immunospecific IgM was detected. The absence of IgM antibody in these three individuals implies that IgM analysis of healthy people may not be as rewarding as analysis of acutely ill patients. Because IgM antibody has a relatively short half-life in vivo compared to IgG, the patient infected several months earlier may show residual IgG antibody activity, but no IgM activity. Work is in progress to determine how long immunospecific IgM circulates after clinical and subclinical group B arbovirus infections.

In order to determine whether other group B arboviruses similarly induce an immunospecific IgM response, the serums of 6 patients with primary dengue fever were subjected to S-DC fractionation. Epidemiological data supported by routine serological tests indicated that 3 of the 6 dengue fever patients selected for study had primary dengue 4 infections. IgM analysis confirmed the existence of IgM reactive only against dengue 4 in these 3 patients, even though each of them had low-titered, cross-reactive serum antibody titer rises to other dengue serotypes and in one case to JEV.

Two of the 6 dengue patients had serological evidence of recent dengue 3 infections; dengue 3 was in addition isolated from the blood of one of the patients. IgM antibody reactive against both D3 and JEV antigens was found in serum of one "dengue 3" patient, while IgM antibody was not detected in the serum of the second patients which was drawn 8 days after onset of fever.

The serum of one patient with primary dengue 2 fever, established by serology and confirmed by recovery of dengue 2 virus from the acute phase blood, was subjected to IgM analysis. IgM antibody reactive to dengue 2 and to dengue 4 antigen was isolated from this patient's serum.

Thus 6 patients provided serological, epidemiological and virological evidence of acute primary dengue virus infections caused by 3 dengue serotypes, and 5 of the 6 had antidengue IgM antibody detectable in their serum. However, 2 of the 5 patients with IgM antibody showed cross IgM reactions, one against another dengue serotype and one to JEV. In summary, IgM analysis identified the specific infecting dengue serotype in only 3 of 6 patients with primary dengue infections.

Finally 2 patients with serological evidence of dengue hemorrhagic fever were studied; dengue 3 virus was isolated from the serum of one. Neither patient had detectable immunospecific IgM antibody in their serums, which confirms the previous finding of Russell et. al., that IgM antibody is not produced after a secondary dengue infection.

It is likely from the work of Japanese investigators that second infections with JE virus can occur, and that IgM is not produced after the second infection. Thus in a group B pre-sensitized patient providing epidemiological, clinical, and serological evidence of an acute group B infection, the absence of detectable IgM antibody may provide indirect evidence that the agent causing the previous and recent infections was the same virus. Conversely, the production of IgM antibody in such a patient may provide serological evidence that the virus producing the acute infection and reacting with the IgM is not the same agent that caused the past infection. This potentially useful diagnostic concept is now being tested.

In summary, IgM antibody was found in the serum of 39 patients with different acute JEV or dengue infections, and in 37 the IgM reacted monospecifically to JEV or to dengue 1-4 antigens (see Table 11). Two patients with primary dengue fever and viremia had cross-reactive IgM antibody against one other antigen in addition to the causative agent. We conclude that the presence of IgM antibody reacting to JEV confirms a recent infection by that virus in individuals experiencing their first group B arbovirus infection and in individuals showing serum antibody cross-reactions to dengue (secondary group B infection). The IgM antibody assay holds promise as a routine laboratory method for the identification of acute JEV infections and perhaps other acute arbovirus illnesses of man. However, it does not hold equal promise as a method for the identification of temporally remote arbovirus infections, nor of repeated infections by the same virus.

Future studies of IgM will aim at confirming its specificity in other group B arbovirus infections, both in man and in animals. In addition investigations already underway are designed to determine how long IgM circulates following clinical and subclinical infections.

Table 8.

Arbovirus Serology: A Comparison of Sucrose Density Gradient Centrifugation with Molecular Sieve and Ion Exchange Chromatography for the Separation of Serum Immunoglobulins.

S-DGC

1. Requires 0.125 ml serum
2. Little or no loss of immunoglobulin in gradient
3. Fractionate 3 to 6 serums per day/centrifuge
4. Acetone-extraction of serum not required
5. Fractionation plus HI testing requires 3 days per serum
6. Reproducible
7. Refractionation required for 5% of serum runs because of incomplete Ig separation

Chromatography

1. Requires 1.0 ml serum
2. Loss of some immunoglobulin in column (ion exchange)
3. Fractionate 1 serum per day/column
4. Acetone-extraction of serum required
5. Fractionation plus HI testing requires 4 days per serum
6. Reproducible
7. Refractionation not required (theoretical)

Table 9. IgM Antibody in the Convalescent Serum of a Patient^(a) with Japanese Encephalitis.

Serum-sucrose fraction no.	Immunoglobulin (b) concentration—mg% IgM	HI Antibody titer											
		JEV		D4		D3		D2		D1			
		C 2ME ^(c)		C 2ME		C 2ME		C 2ME		C 2ME			
1	0	8	<2	2	2	0	0	0	0	0	0	0	0
2	0	16	2	2	2	<2	0	0	0	0	0	0	0
3	25	16	2	4	4	2	0	0	0	0	0	0	0
4	27	13	4	4	4	4	2	0	2	0	0	0	0
5	46	15	4	4	4	4	4	2	2	0	2	0	0
6	19	8	4	4	4	4	2	2	2	0	0	0	0
7	0	10	16	8	8	8	4	4	2	2	2	2	2
8	0	60	>128	64	64	32	32	16	16	16	16	8	8
9	0	140	>128	>128	>128	64	64	32	32	32	32	16	16
10	0	25	128	32	32	16	16	8	8	8	8	16	16
11	0	6	16	16	16	16	16	16	32	32	32	16	16
12	0	32	32	32	32	16	16	16	32	32	32	32	32
Whole serum		2560	1280	1280	1280	640	640	640	640	320	320	320	320

(a) Case number LB—JE—31. Blood drawn 15 days after onset of illness.

(b) Determined by radial immunodiffusion.

(c) C = control aliquot not treated with 2ME.

2ME = aliquot treated with 2ME

Table 10. Absence of IgM Antibody in the Acute Serum of a Patient^(a) with Japanese Encephalitis.

Serum—sucrose fraction No.	Immunoglobulin concentrations—mg% IgM	HI Antibody Titre											
		JE		D4		D3		D2		D1			
		C	2ME	C	2ME	C	2ME	C	2ME	C	2ME		
1	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	6	2	0	2	0	0	0	0	0	0	0	0
3	0	8	2	4	2	0	0	2	2	2	2	2	2
4	20	8	2	2	2	2	0	2	2	2	2	2	2
5	23	8	4	4	2	2	2	4	2	4	2	2	2
6	0	8	4	4	2	2	2	4	2	4	2	4	2
7	0	13	4	8	4	4	4	8	4	4	8	4	4
8	0	110	64	64	64	64	64	64	64	64	64	128	64
9	0	220	>128	>128	>128	>128	64	>128	>128	>128	>128	>128	>128
10	0	35	32	32	32	32	16	32	64	32	64	32	32
11	0	8	16	64	16	8	8	32	32	32	32	8	8
12	0	7.5	16	64	8	16	8	32	32	32	32	8	8
Whole serum		80	40	80	80	80	80	40	40	40	40	20	20

(a) Same patient shown in Table 9. Blood drawn 2 days after onset of illness.

Table 11. Summary of IgM Analysis of Serums from Patients with Group B Arbovirus Infections.

Disease and Patient Population	Serological Pattern	No. patients	IgM antibody reactive against:		
			JEV	D1-4	Neither JEV nor D1-4
I Acute Encephalitis Thai children Chiengmai 1970	a) Secondary-rising(a)	5	5	0	0
	b) Secondary-fixed(b)	1	1	0	0
	c) Primary-fixed(c)	1	1	0	0
II Acute Encephalitis American troops Vietnam 1970	a) Secondary-rising(a)	13	13	0	0
	b) Secondary-rising(b)	7	5	0	2
	c) Primary-rising(d)	2	2	0	0
III Acute psychosis Thai adults Chiengmai 1970	a) Secondary-fixed(d)	1	1	0	0
	b) primary-fixed(c)	4	1	0	3
IV Healthy-Chiengmai Resident-1970	a) Secondary-fixed(b)	3	0	0	3
V Dengue fever (D4) Koh Samui-1967 Bangkok-1969	a) primary-rising(e)	3	0	3(D4)	0
VI Dengue fever (D3) Bangkok-1969	a) primary-rising(e)	2	1(1)	1(D3)	1
VII Dengue fever (D2) Bangkok-1969	a) Primary-rising(e)	1	0	1(D ₂ &D ₄) (2)	0
VII Dengue hemorrhagic fever (D3) Bangkok 1969	a) secondary-fixed(b)	1	0	0	1
	b) secondary-rising(a)	1	0	0	1

(a) ≥ 4 -fold rise in titers to JEV & D1-D4. See table 1a for definition of secondary and primary serological response to JEV

(b) Fixed, elevated titers to JEV and D1-4,

(c) Fixed, elevated titers to JEV

(d) ≥ 4 -fold rise in titers to JEV

(e) ≥ 4 -fold titer rise to one dengue serotype; higher titers to this serotype than to other dengue serotypes or JEV

Footnote

(1) IgM antibody cross-reacting with JEV

(2) IgM antibody cross-reacting with Dengue 4.