

Human Immunoglobulin M Antibody in the Serodiagnosis of Japanese Encephalitis Virus Infections

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INTRODUCTION: The serological identification of a primary group B arbovirus infection can usually be made on the basis of a monospecific antibody response to a type specific virus antigen, measured by the standard serological tests of HI, CF, or Nt. However, the high-titered heterospecific antibody found in serum after sequential infections with group B arboviruses hinders specific identification of the most recent infection. In addition, the rapid anamnestic antibody titer rise in secondary group B infections, together with delays in obtaining the acute phase serum specimens from some patients, often makes it impossible to detect a titer rise in whole serum.

Serum immunoglobulin M (IgM) has been found to be more capable than serum immunoglobulin G (IgG) of distinguishing antigenic differences between certain virus types within non-arbovirus groups (2, 3, 4, 5). Similarly IgM antibody produced in response to group B arbovirus infections of rabbits and guinea pigs was more specific than IgG for the infecting virus, and IgM antibody reacting monospecifically with JEV antigen provided a specific serological diagnosis for first JEV infection in gibbons previously sensitized with dengue. The usefulness of the IgM antibody assay in human dengue infections has recently been reported.

We have encountered many patients in Southeast Asia who have either primary infections with monospecific fixed or falling titers to JEV or dengue, or secondary infections with cross-reactive titers to these viruses. In an attempt to improve the efficiency of serodiagnosis of these infections, we have compared the immunospecificity of isolated IgM HI antibody with the specificity of HI, CF and Nt antibody in whole serum obtained from groups of well-studied patients. This paper describes our experience using IgM antibody analysis in the serodiagnosis of 88 patients hospitalized in Vietnam and Thailand with presumed Japanese encephalitis. We also present the results of IgM antibody titrations on the serum of Thai persons with clinically inapparent JEV infections.

MATERIALS & METHODS:

Patients With Japanese Encephalitis: Acute and convalescent bloods were collected from 2 groups of patients hospitalized with fever and acute encephalitis. The first group consisted of 23 American military personnel, between 18 and 27 yrs of age, hospitalized at the 93rd Evacuation Hospital, Long Binh, Vietnam, from May to October 1970. The presumptive diagnosis of encephalitis was made on the basis of the clinical triad of headache, fever, and central nervous signs and symptoms associated with an abnormal cerebrospinal fluid (CSF), which was sterile for bacteria on culture. A CSF was considered to be abnormal if greater than 10 white cells/cmm were found, or if the protein was elevated above 45 mg%. Convalescent phase sera for arbovirus serology were drawn from each patient 10 to 14 days after the acute phase sera. The time between the onset of illness and the acute phase serum sample ranged from 2 to 17 days. Although 3 patients died, no postmortem brain specimens were obtained for viral isolation attempts; JEV was isolated, however, from the brain of an American soldier dying with encephalitis during the 1969 epidemic of JE which occurred in the Saigon-Long Binh area of Vietnam. All patients had presumably been immunized with 17-D yellow fever virus vaccine prior to their arrival in Vietnam; no patients gave a past history compatible with clinical dengue fever.

Serum pairs from the second group of encephalitis patients, 65 in number, were selected from a serum bank representing more than 120 Thai patients. The patients were hospitalized during a 1970 JE epidemic which occurred in the Chiangmai and Lampang Valleys of Northern Thailand. The presumptive diagnosis of JE was

made using clinical criteria described above. The age of these 64 Thai patients ranged from 3 to 47 yrs, and approximately 2/3 were males. JEV was isolated in this laboratory from the brain of a fatal case, and 13 more isolates of JEV were recovered from vector mosquito pools collected in Chiangmai during the epidemic. Detailed epidemiological and clinical descriptions of these and additional patients hospitalized during the 1970 epidemic will be published elsewhere. Acute phase serum specimens were drawn 1-24 days after onset of disease, with a median of 4 days. Convalescent phase sera were drawn from each patient 3 to 72 days after the acute specimen, with a median interval of 14 days.

Subjects with Inapparent Infections: Sera obtained from two groups of healthy Thai persons residing in Chiangmai Valley were tested for evidence of recent JEV or group B infection. The first group consisted of more than 70 family members of hospitalized JE patients. These family members volunteered for serial bleedings performed prospectively over 2 to 8 week intervals during the 1970 epidemic. Sera from eleven family members with evidence of inapparent infections were selected for IgM analysis. Another group of 31 subjects was selected from over 400 Chiangmai valley villagers and Chiangmai city schoolchildren who participated in a study designed to monitor the incidence of inapparent JEV infection in Chiangmai valley in 1970. They were bled at approximately 3 month intervals. Persons in Chiangmai valley reside in an area where JEV, dengue and Tembusu viruses are endemic.

Serological tests: HI and CF tests were performed in microtiter plates as described previously, using 8 units of HA arbovirus antigens for the HI test, and 8 CF units of antigen and 2 units of complement for the CF test. The Nt test previously described was employed with minor modifications, in that plaques in JEV and dengue infected cultures were counted on days 5 and 6, respectively.

The following virus strains were used for the 3 serological tests: JEV (Nakayama), dengue 1 (Hawaii), dengue 2 (New Guinea "C"), dengue 3 (H-87) and dengue 4 (H-241). In addition, Tembusu virus (LGLT-377) and Wesselsbron virus (BKM 367-66) were used in HI tests of sera from 11 individuals with inapparent infections.

Treatment of whole serum with 2-mercaptoethanol: One part of a 1:10 dilution of 2-ME in borate saline (0.2 molar), pH 9.0, was added to 9 parts of goose-erythrocyte adsorbed, acetone-treated serum. The mixture was incubated at 37°C for 30 minutes and then placed at 4°C for 30 minutes. The 2-ME treated and untreated aliquots of the same serum were then diluted simultaneously for the HI test run against 8-16 HA units of D 1-4 and JEV antigens. If the HI antibody titer after treatment was reduced to or below one-fourth of the titer before treatment, the serum was judged to contain 2-ME sensitive (IgM) antibody.

Serum Fractionation by Sucrose Density Gradient Centrifugation: The fractionation of serum followed standard SDGC methodology. Briefly, 0.125 ml of heat-inactivated serum was diluted with normal saline to 0.25 ml, adsorbed with goose erythrocytes, and then 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 were collected in 0.3 ml aliquots, while fractions 8 thru 12 were collected in 0.5 ml volumes. Each fraction was divided into 2 aliquots. One aliquot was treated with 2-ME (0.135 ml sucrose fraction plus 0.15 ml of 0.2 molar 2-ME) for 30 min at 37°C and then for 30 min at 4°C; the second aliquot was treated with 0.15 ml buffer similarly. The untreated (control) and 2-ME treated aliquots were then diluted for HI titration against 8-16 units of HA arbovirus antigens. Serum from patients with encephalitis were routinely tested against JEV & D1-4 antigens; healthy persons were tested against JEV, one or more dengue serotypes, Wesselsbron, and Tembusu antigens. The concentration of IgM and IgG in the 12 serum-sucrose fractions of each of 12 sera was determined by radial immunodiffusion in agar ("Immunoplates" Hyland Laboratories, Los Angeles, Calif). All of the IgM detectable by radial immunodiffusion was concentrated into serum-sucrose fractions 3, 4 & 5, and occasionally into fractions 2 thru 6. Between 80-100% of the detectable IgG was found in fractions 7-12, while the remainder, if any, was detected in fractions 3-6, and accounted for 0%-40% of the immunoglobulins contained in these early fractions (3, 4, 5); these were always selected without pooling for 2-ME treatment and HI titrations; the remaining 9 fractions were discarded. As previously

reported the non-specific HA inhibitors were isolated in the top of the sucrose gradient (fraction 12); therefore sera were not extracted with acetone before HI testing.

RESULTS:

Serological criteria; whole serum: Encephalitis patients were divided into 2 serological groups on the basis of their whole serum HI & CF antibody responses to JEV and D1-4 antigens. The first group had titer rises (≥ 4 -fold) to JEV alone, or to JEV & D4 with higher JEV convalescent titers. These patients were presumed to have had a recent primary immune response to their first exposure to group 2 arbovirus (JEV). The second group were considered to have had a probable recent JEV infection secondary to prior group B arbovirus experience. This secondary-type serological pattern was characterized by an anamnestic antibody response, with high convalescent titers (generally $\geq 1:640$ by HI and $\geq 1:64$ by CF) to JEV and to 2 or more dengue serotypes; the JEV convalescent titer was usually ≥ 4 -fold higher than any one of the 4 dengue serotypes. Serological patterns varied according to the test employed. The one test providing the most definitive serological result, i.e., monospecific instead of heterospecific, and rising rather than fixed or falling titers, was recorded. Patients with monospecific fixed or falling titers against JEV in paired serum spaced greater than 7 days apart were considered to have been infected once with JEV at some unspecified time in the past. Patients with heterospecific fixed or falling titers to JEV and to two or more dengue serotypes were considered to have been infected more than once by group B arboviruses in the past. Representative primary and secondary serological patterns (Table 1) illustrate that any one of the three serological tests provided a specific diagnosis of a recent or remote JEV primary infection, whereas none of the 3 serological tests identified the type-specific group B arbovirus in secondary infections.

Treatment of whole serum with 2-mercaptoethanol: In an attempt to confirm JEV as the specific group B arbovirus responsible for acute encephalitis in patients with secondary infections, we tested for the presence and reactivity of IgM antibody by treating whole serum with 2-ME. 2-ME labile antibody, reactive against JEV but not D1-4, was detected in the sera of 6 of 7 Thai and American patients with primary JEV infections, but in none of 13 patients with secondary infections. These results indicate that IgM antibody does appear in the majority of patients with a primary immune response to a recent JEV infection, and that the antibody is specific for JEV. The inability to detect IgM antibody after secondary infections may have been the consequence of high-titered IgG antibody masking lower-titered IgM antibody in whole serum. We therefore isolated IgM and IgG from whole serum by sucrose density gradient centrifugation (SDGC).

Serological Criteria: SDGC Fractions of whole serum: Virus-specific IgM antibody was considered present in serum-sucrose fractions of whole serum when the HI titer against one of the HA antigens fell after 2-ME treatment. The fall was considered significant when 2-ME reduced the HI titer to $< 1/8$ of the control titer in any one of the 3 IgM rich fractions, or to $< 1/4$ of the control in 2 or more fractions. These criteria are weighted in favor of eliminating false positive IgM results. In a small number of sera, we noted a 4-fold HI titer drop in one IgM-rich fraction and a 2-fold reduction in 2 additional IgM fractions after 2-ME treatment. Such sera were considered to contain "trace" IgM antibody activity against the antigen(s) in question. The IgM antibody titer was considered to rise or fall significantly when the titer between acute and convalescent sera differed ≥ 2 -fold in at least 2 fractions, or ≥ 4 -fold in at least one fraction. Low-titered IgM antibody was lost after the unavoidable 10-fold dilution of serum in the sucrose gradient. This dilutional loss of IgM antibody activity, resulting in false negative results, was noted particularly in serum having JEV HI titers of $< 1:40$.

Repeated testing of IgM antibody negative and positive sera (15 sera refractionated and titered 2 to 6 times) resulted in reproducible titrations in greater than 95% of runs. The SDGC procedure was precise in that doubling the serum volume placed on the gradient resulted in a 2-fold increase of IgM and IgG antibody titers. Storage at -20°C for 12 months, and as many as 4 freeze-thaw cycles, produced no significant loss of JEV IgM antibody activity.

Serum IgM antibody after Japanese Encephalitis: The paired sera from the 4 patients shown in Table 1 were fractionated and the results are listed in Table 2. The 2-ME labile HI antibody limited to fractions 3-5, which contained all immunoprecipitable IgM, reacted with JEV but not with D1-4. In contrast, HI antibody in fractions 7-12 (not shown) reacted in high titers (1:16 to > 1:128) with both JEV and dengue antigens, was 2-ME resistant, and was IgG by radial immunodiffusion. We conclude that the SDGC procedure partially or completely isolates IgM from IgG and thereby permits measurement of the IgM antibody titer. Furthermore, the results in Table 2 indicate that JEV-specific IgM antibody is produced not only in primary JEV infections, but after JEV infections in patients with previous group B arbovirus experience as well. The IgM antibody titers in paired sera may be rising (Patients A and C), fixed (not illustrated), or falling (Patients B and D). The contamination of IgM by IgG probably accounts for the 2-ME resistant antibody found in some IgM-rich fractions. This contamination was observed more often in high-titered sera (Patients C and D) than in low-titered sera (Patients A and B). The presence of serum IgM immunoreactive against JEV in these patients with acute encephalitis provides additional serological confirmation of a recent JEV infection.

A further comparison of whole serum and IgM antibody patterns in 23 American and 65 Thai encephalitis patients is presented in Table 3. Twenty-one American troops had IgM antibody reactive only against JEV, and 19 of these 21 had rising IgM titers. One patient (E, Table 4) had rising IgM convalescent antibody titers cross-reacting with JEV (> 1:128) and D4 (1:8). IgM antibody was not detected in a final patient, a female nurse stationed in Saigon, who had low-fixed whole serum HI titers of 1:20-1:40 against JEV and D4. Thus rising titers in conventional serological tests specifically confirmed a recent JEV infection in only 7 American patients (30%). HI titration of isolated IgM confirmed the diagnosis in 19 patients (83%), and led to a presumptive diagnosis of JEV in 3 more persons with fixed, falling or weakly heterospecific IgM titers.

JEV IgM antibody was found in 41 of 65 hospitalized Thai children and adults (Table 3). Thirty-nine of these 41 persons had JEV monospecific activity, and 31 of these 39 showed rising IgM titers; the remaining 8 patients had fixed or falling IgM titers. Most IgM negative patients fell into the secondary infection group; possible reasons for this disparate distribution of negative patients will be discussed later. Two of the 41 Thai patients displayed cross-reactive IgM antibody titers (Table 4). The high JEV and low D4 IgM titers in patient F suggest a recent JEV infection. In patient G, the unusual combination of high-titered, cross-reactive IgM against JEV and D1, 2 and 3 precludes the presumptive serodiagnosis of JE.

As seen in Table 3, rising titers in conventional serological tests in Thai patients specifically confirmed a recent JEV infection in 19 persons (29%), while the diagnosis was confirmed by JEV-specific, rising IgM antibody titers in 31 persons (48%). In addition, the JEV IgM titers were fixed in 1 patient, falling in 7, and weakly heterospecific in 1 (patient F; Table 4), providing a presumptive or confirmed diagnosis of JEV in 40 cases (61%).

IgM antibody reacted monospecifically with JEV in 60 of 63 Thai & American patients whose sera contained IgM antibody activity.

The diagnostic efficacy of each of the 4 serological tests used in this study is compared in Table 5. Only patients with monospecific rising titers to JEV are considered positive. Twice as many presumed cases of JE were confirmed as recent JEV infections by IgM analysis than by any other single serological test. The apparent superiority of IgM analysis could be increased further if the 12 patients with fixed, falling or low titered heterospecific IgM patterns are included.

Serum IgM antibody after inapparent JEV infections: It was of interest to determine whether serum obtained from persons with inapparent JEV and secondary group B arbovirus infections contained JEV-specific IgM antibody. Thirty-six sera from 31 apparently healthy Chiangmai villagers and urban school children, with evidence of subclinical infections detected serologically at 12-16 week interval bleedings, were fractionated by SDGC (Table 6). Only one serum from a Chiangmai city school child contained trace amounts of

antibody to D2. The 35 remaining sera contained no detectable IgM antibody. These results raised the possibility that IgM antibody may not be produced after inapparent JEV infections.

In order to further investigate this possibility, we fractionated sera obtained from 11 additional Chiangmai villagers with inapparent JEV or group B infections. These infections were diagnosed by antibody titer rises in serial sera drawn prospectively at 2 to 8 week intervals. Five of the 11 subjects had primary rising titers; 4 of these produced IgM antibody reactive with JEV but not D1-4, Tembusu, or Wesselsbron viruses (Table 6). Virus-specific IgM antibody is therefore produced in inapparent JEV infections. In contrast to the findings in encephalitis patients, no IgM antibody activity was detected in 6 serum pairs showing primary falling or secondary infection patterns.

SUMMARY & CONCLUSIONS:

Paired sera, obtained from 88 American and Thai patients hospitalized with presumed Japanese encephalitis, were tested by the standard serological techniques of hemagglutination inhibition (HI), complement fixation, and plaque reduction neutralization. On the basis of these tests, 35 patients had recent or remote primary JEV infections, while 53 patients had secondary group B arbovirus infections characterized by high cross-reactive antibody titers to JEV and to dengue virus serotypes 1-4. Immunoglobulin M (IgM) HI antibody, isolated from whole serum by sucrose density gradient centrifugation, reacted with JEV but not dengue viruses in 31 secondary cases and in 29 primary infection patients. IgM antibody reacted monospecifically with JEV in 60 of 63 patients whose serum contained detectable IgM activity. Moreover, rising JEV IgM antibody titers were found in 6 of 25 patients having fixed or falling whole serum antibody titers. Altogether, 57% of patients were confirmed as recent JEV infections by rising IgM antibody titers, while only 26% were confirmed by conventional serological tests. On the basis of these findings more precise serological criteria have been formulated which utilize whole serum and IgM antibody titrations together for the diagnosis of JEV infections.

Table 1. Representative serological patterns in whole sera from patients with fever and encephalitis.

Patient	Day of Disease	Reciprocal serum antibody titer																		Sero Pattern ^{xx}	
		HI						CF						Nt							
		JEV	D4	D3	D2	D1		JEV	D4	D3	D2	D1		JEV	D4	D3	D2	D1			
A	6	40	0 ^{xxx}	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	1°R	
18 yr male American	12	320	40	20	20	20		32	0	0	0	0	0	0	0	0	0	0	0	0	1°R
B	4	320	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0		1°F _x
9 yr male Thai	16	320	20	0	0	0		4	0	0	0	0	0	0	0	0	0	0	0	0	1°F _x
C	4	320	640	320	160	320		8	16	16	16	16	16	16	16	40	300	640	550		2°R
11 yr female Thai	13	2560	1280	1280	320	640		64	64	64	64	64	64	64	320	100	640	2200	2000		2°R
D	24	5120	1280	2560	640	5120		128	128	32	64	64	64	64	350	2300	40	800	1200		2°F _x
16 yr male Thai	35	2560	1280	2560	1280	2560		16	128	32	32	64	64	270	900	40	800	2560			2°F _x

xx For this and all subsequent tables; 1° = primary JEV infection,

2° = secondary group B arbovirus infection, R = rising titer (≥ 4 -fold),

F_x = fixed titer, F₁ = falling titer (≥ 4 -fold) by HI or CF.

xxx For this and all subsequent tables; 0 = $<1:20$ for HI, $<1:4$ for CF, $<1:10$ for Nt.

Table 2. IgM antibody titration of paired sera from encephalitis patients with primary JEV & secondary group B arbovirus infections.

Pt Whole ^{xx} Serum Serological Pattern	Day of Disease	Serum# Sucrose Fraction	Reciprocal HI antibody titer									
			JEV		D4		D3		D2		D1	
			C*	2-ME**	C	2-ME	C	2-ME	C	2-ME	C	2-ME
A 1°R	6	3	2	0	0	0	0	0	0	0	0	0
		4	2	0	0	0	0	0	0	0	0	0
		5	2	2	0	0	0	0	0	0	0	0
	12	3	8	0	0	0	0	0	0	0	0	0
		4	16	0	0	0	0	0	0	0	0	0
		5	64	0	0	0	0	0	0	0	0	0
B 1°F _x	4	3	8	2	2	2	2	2	0	2	2	
		4	16	0	2	0	2	2	2	2	2	
		5	16	2	2	0	4	4	2	2	4	
	16	3	8	0	2	0	2	2	2	0	2	0
		4	16	2	2	0	2	2	2	0	2	0
		5	4	2	0	0	4	8	2	0	2	2
C 2°R	4	3	2	2	2	4	4	4	4	2	4	
		4	4	2	2	4	4	4	4	4	4	
		5	4	2	2	4	8	4	4	4	4	
	13	3	32	8	4	4	4	4	4	4	8	8
		4	64	8	4	4	4	4	4	4	8	8
		5	32	8	4	4	4	4	4	4	8	8
D 2°F _x	24	3	32	8	8	8	16	16	8	8	8	8
		4	32	8	8	8	16	8	8	8	8	8
		5	32	8	8	4	16	8	8	8	16	8
	35	3	16	8	8	8	16	16	8	16	8	8
		4	32	8	8	8	16	8	16	16	16	8
		5	16	8	8	8	16	8	16	16	16	8

xx Antibody titers listed in Table 1.

Fractions containing IgM as detected by radial immunodiffusion.

*C = aliquot of serum-sucrose fraction first treated with buffer

**2-ME = aliquot of serum-sucrose fraction first treated with 2-mercaptoethanol.

Table 3. Comparison of whole serum and serum IgM antibody patterns in Americans and Thais hospitalized with presumed Japanese encephalitis.

Patients	Whole serum antibody pattern	Number of serum pairs	IgM HI Antibody Reactivity				
			JEV ^x			JEV + D ^{xx}	Negative [#]
			R	F _x	FI		
American	1° R	7	6	0	0	1	0
	2° R	10	9	1	0	0	0
	2° F _x	6	4	1	0	0	1
	Sub -- total	23	19	2	0	1	1
Thai	1° R	19	16	0	2	0	1
	1° F _x	9	2	1	2	0	4
	2° R	27	13	0	1	1	12
	2° F _x	7	0	0	0	1	6
	2° FI	3	0	0	2	0	1
	Sub -- total	65	31	1	7	2	24
	Total	88	50	3	7	3	25

x JEV — specific IgM Ab in one or both sera of a pair

xx IgM antibody cross — reactive with JEV and one or more dengue serotypes in one or both sera of a pair.

IgM antibody not detected

Table 4. Cross-reactive IgM antibody patterns in three patients with encephalitis.

Patient Serological Pattern Whole Serum	Day of Disease	Serum Sucrose Fraction+	Reciprocal HI antibody titer+										
			JEV		D4		D3		D2		D1		
			C	2 ME	C	2 ME	C	2 ME	C	2 ME	C	2 ME	
E 24 yr male	6	3	2	0	0	0	0	0	0	0	0	0	0
		4	8	0	2	0	0	0	0	0	0	0	0
		5	4	0	4	0	0	0	0	0	0	0	0
American 1°R	16	3	32	0	4	0	0	0	0	0	0	0	0
		4	> 128	0	4	0	0	0	0	0	0	0	0
		5	> 128	0	8	0	2	0	2	0	0	0	0
F 18 yr male	2	3	16	8	8	4	8	2	8	4	4	4	4
		4	16	8	8	4	8	4	4	4	4	4	4
		5	16	8	8	2	8	4	8	4	4	4	4
Thai 2°F _x	19	3	16	4	4	0	4	2	2	2	2	2	2
		4	32	4	8	0	4	2	2	2	2	2	2
		5	16	4	4	0	4	2	2	2	2	2	2
G 47 yr female	5	3	2	0	0	0	2	2	2	2	2	0	0
		4	4	0	0	0	4	2	2	2	4	2	2
		5	2	0	0	0	4	4	2	2	2	2	2
Thai 2°R	22	3	32	8	16	8	16	8	16	4	32	4	4
		4	64	8	16	8	> 128	8	64	4	64	4	4
		5	32	8	16	8	16	8	16	4	16	4	4

+ See footnotes Table 2.

Table 5. Comparison of four serological tests used in the diagnosis of Japanese Encephalitis.

Patient Population	No. Patients with ≥ 4 -fold JEV monospecific antibody titer rises			
	Whole Serum		Nt	Isolated IgM
	HI	CF		HI
Thai	^{xx} $\frac{19}{65}$	$\frac{16}{61}$	$\frac{0}{3}$	$\frac{31}{65}$
American	$\frac{2}{23}$	$\frac{7}{23}$	$\frac{3}{9}$	$\frac{19}{23}$
Total	$\frac{19}{88}$	$\frac{23}{84}$	$\frac{3}{12}$	$\frac{50}{88}$
Total Percent Positive	22 %	28 %	25 %	57 %

xx No serum pairs positive
No serum pairs tested

Table 6. IgM antibody production in inapparent JEV and group B arbovirus infections.

Patient Population	Serum HI or CF Antibody titers	Number of Persons Contributing Serum Pairs or Triplicates	Number of Persons with JEV IgM Antibody
Chiangmai valley villagers and urban school children ^x	1°R 1°F 1°F ^x 2°R 2°F 2°F ^x	10 1 1 15 2 2	0 0 0 0 0 0
Chiangmai valley villagers ^{xx}	1°R 1°F 2°R 2°F ^x	5 3 1 2	4 0 0 0

x Bled at 12-16 week intervals.
xx Bled at 2-8 week intervals.