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Detection of Japanese Encephalitis Virus Immunoglobulin M Antibodies in Serum by Antibody Capture Radioimmunoassay

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An assay for detecting human immunoglobulin M (IgM) antibodies to Japanese encephalitis (JE) virus was developed by using the antibody capture solid-phase radioimmunoassay approach (JE IgM ACRIA). Heavy-chain-specific goat anti-human IgM was first bound to the wells of a polyvinyl microtiter plate, and successive steps involved sequential binding of test sample IgM, acetone-extracted mouse brain JE antigen, and ^{125}I-labeled flavivirus hyperimmune human IgG. Among 20 patients hospitalized in Bangkok with clinical diagnoses of acute encephalitis, and with acute flavivirus infections proven by hemagglutination inhibition (HAI) serology, 16 had detectable (positive/negative [P/N] ratio, >3.0) JE IgM ACRIA antibodies in the acute-phase serum specimen, and 19 had such antibodies in the convalescent-phase serum specimen. Convalescent patient sera regularly had higher P/N values than the corresponding acute-phase sera (mean ± 1 standard deviation = 13.0 ± 9.3 with acute-phase sera and 25.8 ± 19.6 with convalescent-phase sera). JE virus-infected patients with HAI serological responses indicative of a primary flavivirus infection had higher JE IgM ACRIA P/N responses than did those patients whose serological response indicated past exposure to other flaviviruses. None of 70 serum specimens from healthy Thai adults and children with serum JE HAI antibodies had detectable JE IgM ACRIA activity (P/N ratios all ≤3.0). Biological false-positives with low P/N ratios (range, 3 to 15) were found in sera from patients with acute or recent infections with flaviviruses other than JE virus but could be differentiated by the fact that these sera gave higher P/N ratios with homologous antigens than with JE virus. False-positive reactions with low P/N ratios (range, 3 to 6) due to serum rheumatoid factor activity were differentiated by testing with control antigen. The JE IgM ACRIA technique permits a rapid, accurate diagnosis of acute JE virus infections in both patients with and those without previous exposure to other flaviviruses.

Existing laboratory methods for the diagnosis of acute Japanese encephalitis (JE) virus infections have limited clinical usefulness. First, JE virus can virtually never be recovered from the blood or cerebrospinal fluid of encephalitis patients (10), so that an etiological diagnosis must be made by conventional serological methods with their inherent 1- to 2-week delay. Second, the widespread prevalence of flaviviruses other than JE in many countries renders diagnoses based on hemagglutination inhibition (HAI) seroconversions nonspecific due to the extensive serological cross-reactions between members of this group (11). Finally, probably because the encephalitic phase of JE infections occurs relatively late in the illness, in our experience many patients have already seroconverted by the time the acute-phase serum is obtained, thereby making it impossible to demonstrate a fourfold antibody rise with routine HAI procedures.

Immunoglobulin M (IgM) antibodies with specificity to JE virus antigens have been detected by sucrose density gradient fractionation of acute- and convalescent-phase sera of encephalitic patients; these IgM antibodies persist for less than 3 months in most patients (3-5, 7, 12). Unfortunately, the need to ultracentrifuge the specimens has limited the applicability of this approach.

We recognized that the reverse solid-phase immunoassay technique introduced by Duermeyer et al. (2) for detection of IgM anti-hepatitis A virus antibodies could probably be modified to detect anti-flavivirus IgM. We report here our efforts to develop this assay.

MATERIALS AND METHODS

Patient sera. Paired acute- and convalescent-phase sera which had been sent to our laboratory from neighboring hospitals in Bangkok formed the main study battery (group I). Although detailed clinical

information was not available to us on all 43 study patients, all were assigned clinical diagnoses of acute encephalitis. Thirty-six were patients at the Bangkok Children's Hospital, four were patients at the Royal Thai Army Hospital, and three were patients at other local hospitals. All but one were children ages 2 to 12 years; one was 26 years old. Three cases were fatal. On the basis of HAI antibody titrations on acute- and convalescent-phase sera, these 43 patients were each assigned to one of four categories. Group IA contained 12 patients diagnosed as having primary flavivirus infections (fourfold or greater antibody titer rise to JE antigen; convalescent-phase titers to D1, -2, -3, and -4 and to JE virus (all $\leq$1:640). Group IB consisted of eight patients with secondary flavivirus infections (antibody present in acute-phase serum at a titer of $\geq$1:20 to at least one antigen; acute- or convalescent-phase titer to at least one antigen of $\geq$1:1,280). In group IC, 10 patients were antibody negative without seroconversion (antibody negative in both acute- and convalescent-phase sera). Group ID contained 13 patients for whom the diagnosis was uncertain, in that these patients had antibody in both acute- and convalescent-phase sera but showed no fourfold titer rises (all titers were <1:1,280 in both acute- and convalescent-phase sera).

Group II consisted of sera taken from 20 healthy adult Thai laboratory workers. All had broadly cross-reactive serum HAI antibodies to JE virus and all four dengue serotypes, and nine had detectable (50% plaque reduction neutralization antibody titer of $\geq$1:10) neutralizing antibodies to JE virus. Group III consisted of sera drawn in January 1978 from 60 healthy school children ages 5 to 12 attending a lower-socioeconomic-group public school in Bangkok. Sera from 50 of these children (group IIIA) had unchanging titers of broadly reactive HAI antibodies in specimens obtained in both June 1977 and January 1978. Another 10 sera (group IIIB) were selected which showed evidence of an acute primary dengue virus infection (seroconversion) during the same interval. Group IV consisted of 20 sera taken from children approximately 2 weeks after the onset of acute symptomatic dengue infections documented by HAI serology. Ten patients (group IVA) had a primary flavivirus-type HAI response pattern; another 10 (group IVB) had a secondary flavivirus-type HAI response pattern. The infecting dengue virus types were not determined in these cases. Group V consisted of 11 sera found to be positive for rheumatoid factor (RF) by the latex agglutination technique at the Royal Thai Army Hospital and kindly provided by Kanoklada Barlee. None of the patients from whom these sera were obtained had a clinical diagnosis of encephalitis.

Monkey sera. Two healthy adult male rhesus monkeys (*Macaca mulatta*) lacking serum HAI antibodies to dengue and JE viruses were inoculated intravenously with 1.0 ml of a fresh 20% mouse brain suspension of high-mouse-passage Nakayama strain JE virus containing 10^9 PFU/ml and 5,120 HA units after sucrose acetone extraction. One monkey (G348) was pretreated on day -1 and again on day 2 intravenously with inactivated *Bordetella pertussis* vaccine as part of another experiment; the other (G346) was given only JE virus. Blood was drawn from both animals on days -1, 0, 10, 15, 30, 60, and 150, and whole sera were tested for HAI antibodies to JE virus and IgM anti-JE virus by the antibody capture solid-phase radioimmunoassay (ACRIA) method. Both monkeys remained healthy throughout the study period; neither developed signs of central nervous system infection. Viremia was detected by the mosquito inoculation technique (8) on day 2 after inoculation in both animals and on day 4 in monkey G346. No viremia was detected on day 6, 8, or 10.

Measurement of total serum IgM concentrations. Total serum IgM was measured by a sandwich-type solid-phase enzyme-linked immunoassay (9), using commercial goat anti-human mu chain (Hyland, Division of Travenol Laboratories, Inc., Costa Mesa, Calif.) both as the solid-phase bound antibody and as the alkaline phosphatase (Sigma type VII; Sigma Chemical Co., St. Louis, Mo.) labeled antibody. Reference dilutions of a known commercial standard serum IgM preparation (Hyland) were run during each assay. Hydrolysis of nitrophenylphosphate substrate was read as the change in the optical density at 410 nm, and the IgM concentrations were determined by interpolating onto the standard curve.

Sucrose density gradient fractionation of sera. Volumes (0.3 ml) of selected sera were subjected to rate zonal ultracentrifugation through 4.8-ml continuous 10 to 40% sucrose gradients for 18 h in an SW50.1 rotor in a Beckman L-5 ultracentrifuge at 39,000 rpm.

Ten fractions, each approximately 0.5 ml, were obtained by puncture of the bottom of the fraction tube. By immunodiffusion against heavy-chain-specific goat antisera, IgM was always confined to fractions 2, 3, and 4, whereas IgG and IgA were found in fractions 5 through 10, with peaks in fraction 7 or 8.

HAI serology. HAI serology was performed according to the method of Clarke and Casals (1) as modified for the microtiter system. All sera were extracted twice with acetone and absorbed with goose erythrocytes before testing. The antigens used were sucrose and acetone extracts of pooled infected suckling mouse brains.

The virus strains and suckling mouse passage levels used as routine antigens were dengue type 1, Hawaii strain, passage 17; dengue type 2, New Guinea C strain, passage 32; dengue type 3, H87 strain, passage 27; dengue type 4, H241 strain, passage 33; and JE, Nakayama strain, passage 17. The lowest dilution of whole extracted serum tested was 1:10.

Serum specimens fractionated by sucrose density gradient centrifugation were absorbed with goose erythrocytes but were not acetone extracted; the gradient fractionation removed all nonspecific inhibitors to the serum-gradient interface. The lowest dilution of gradient fractions tested was 1:4. Samples of gradient fractions were tested both in the absence of 2-mercaptoethanol (2-ME; Eastman Organic Chemicals, Rochester, N.Y.) and after 60 min of exposure to 0.1 M 2-ME at room temperature.

Preparation of hyperimmune anti-flavivirus IgG. A stock of hyperimmune anti-flavivirus sera was prepared by pooling 1- to 2-ml volumes of 10 convalescent-phase sera (14 days after onset) from patients with dengue hemorrhagic fever with secondary type (high-titered, broadly reactive) antibody responses. The pool had an HAI titer of 1:5,120 against JE virus and 1:5,120 against D4.

Hyperimmune anti-flavivirus IgG was prepared from the stock serum pool by repeated precipitation

with 33% ammonium sulfate, exhaustive dialysis of the redissolved precipitate against 0.005 M sodium phosphate buffer, pH 8.0, binding to a DEAE A-50 ion-exchange resin (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) in the same buffer, elution from the resin with 0.04 M sodium phosphate buffer dialysis against 0.25 M sodium phosphate buffer, pH 7.4, and concentration by pressure dialysis to 5 mg/ml. This reagent was stored frozen at −20°C until labeling.

Labeling of IgG. For use in radioimmunoassays, IgG was labeled with ^{125}I by a modification of the chloramine-T method (6). In the reaction mixture, 500 MCi of ^{125}I (ICN, Irvine, Calif.) was added to 20 μg of IgG. The mixture was chromatographed over a 10-ml Sephadex G-25 coarse (Pharmacia) column and stabilized with phosphate-buffered saline (PBS), pH 7.4, containing 0.1% sodium azide, 2 mg of KI per ml, and 1% bovine serum albumin (BSA; Sigma). Typically, 50 to 75% of the counts per minute eluted in the void volume with the protein. Under these conditions, one labeling procedure provided enough labeled IgG for 10 to 12 plates or 600 to 700 single determinations. ^{125}I-labeled IgG was always used within 2 weeks after radiolabeling.

Performance of JE IgM ACRIA: immunoassay. The JE IgM ACRIA was performed in five basic steps.

(i) Step 1: sensitization. The central 60 wells of a disposable 96-well polyvinyl U-bottom microtiter plate (220-24; Dynatech Laboratories, Inc., Alexandria, Va.) were sensitized by putting 100 μl of an optimal dilution of goat anti-human mu chain (Hyland) (usually 1:800; see below) in 0.006 M sodium carbonate buffer, pH 9.5, per well for 4 h at room temperature. The plates were then washed three times with 0.05% Tween-80 (Sigma) in PBS (PBS-T).

(ii) Step 2: test sample. Fifty microliters of the test sample diluted in 0.5% BSA in PBS (PBS-BSA) was added to each well and incubated for 2 h at room temperature. For routine purposes, sera were tested at a 1:100 dilution. No extraction or heat inactivation of sera was required. The plates were then washed three times with PBS-T. (Special care was taken during this wash to avoid possible cross-contamination of well contents during the washing procedures; even slight spillage of IgM with anti-JE virus activity into wells lacking IgM may result in false-positive results.)

(iii) Step 3: antigen. Fifty microliters of antigen diluted in a solution of 80% PBS-BSA plus 20% acetone-extracted normal human serum (dengue and JE virus antibody negative) was added per well and incubated overnight at 4°C. For routine purposes, 20% sucrose-acetone-extracted mouse brain antigen (the same antigen as used at a dilution of 1:100 [approximately 50 HA units] was used. The plates were then washed five times with PBS-T.

(iv) Step 4: label. Twenty-five microliters of ^{125}I-labeled hyperimmune anti-flavivirus IgG diluted in 20% acetone-extracted normal human serum in PBS-BSA to give 150,000 to 200,000 cpm/25 μl was added per well and incubated for 2 h at 35°C. The plates were then washed seven times with PBS-T.

(v) Step 5: counting. Individual wells were cut out and counted for 1 min in a Packard model 5375 Auto-Gamma spectrometer system. Two negative control sera were tested at a dilution 1:100 on every plate. All control and test specimens were tesed in duplicate. Positive/negative (P/N) ratios were expressed as the mean counts per minute of the two test wells divided by the mean counts per minute of the four negative control wells.

RESULTS

Preliminary experiments. A series of preliminary experiments was performed in which the reagent dilutions, incubation times, and incubation temperatures were varied to determine the optimal conditions for the assay.

(i) Sensitization step. Dilutions of sensitizing antiserum (step 1) between 1:200 and 1:1,600 provided near optimal sensitization. Little difference was found between incubation times of 1, 4, and 16 h and between temperatures of 25 and 37°C.

(ii) Test sample step. Under standardized test conditions, the concentration of IgM in a typical human serum specimen (1 to 2 mg/ml) was found to be 10^2- to 10^3-fold greater than the concentration required to saturate (≥80%) all of the goat anti-mu chain receptor sites bound to the plastic surface.

When the plates were sensitized with an optimal dilution of goat anti-mu chain antiserum, the minimal concentration of human IgM that could be detected bound to the plates by ^{125}I-labeled goat IgG anti-human mu chain was 10 ng/ml (equivalent to a 10^{-5} dilution of serum); at concentrations above 1,000 ng/ml (10^{-3} dilution of serum), no further increases in bound counts per minute were observed.

(iii) Antigen-binding step. Satisfactory results were obtained with JE virus antigens from several sources, including sonicated fresh mouse brain suspensions, LLC-MK2 cell cultures, and *Toxorhynchites splendens* mosquito suspensions. However, we selected sucrose- and acetone-extracted mouse brain suspensions for routine use, as this antigen is typically high titered, safe because the virus is rendered inactive by the extraction procedure, and convenient because this is the same antigen routinely prepared in our laboratory for use in HAI assays. Concentrations of antigen as low as 0.1 to 0.2 HA units were detectable. The optimal antigen concentration was found to be a 1:100 dilution of stock antigen (approximately 50 HA units). The time and temperature of the antigen incubation step were also found to be important. At low temperature (4°C), increased duration of this step led to improved P/N ratios, but at higher temperatures (25 and especially 37°C), increased duration of the antigen-binding step beyond 1 to 2 h led to lowered P/N ratios. An overnight incubation of 4°C was found to give optimal results.

Assay of sucrose density gradient fractions by JE IgM ACRIA. Figure 1 shows the results of HAI and JE IgM ACRIA assays on fractions

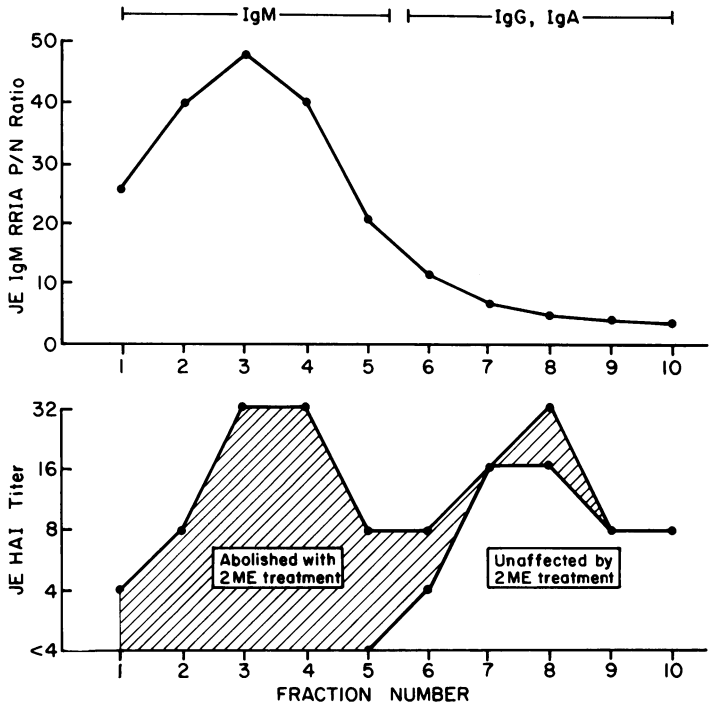

FIG. 1. HAI and JE IgM ACRIA activity in sucrose density gradient fractions of a representative convalescent-phase serum specimen from a patient with acute encephalitis.

from sucrose density gradient fractionation of a serum specimen from a patient with a primary JE virus infection. The peak of activity detected by JE IgM ACRIA corresponded to the peak of 19S, 2-ME-sensitive activity, and not with the peak of 7S, 2-ME-insensitive activity.

Assay of monkey sera after experimental inoculation with JE virus. Figure 2 shows the time course of the appearance of IgM anti-JEV as detected by the ACRIA method compared with total serum HAI antibody titers. The ACRIA response was both more rapid (peaking on day 10 to 15 postinoculation) and more transient (dropping to near baseline by 60 days and disappearing before 150 days) than the whole-serum HAI seroresponse.

Definition of positive cut-off value. The upper limit of normal P/N ratios was arbitrarily defined as the mean + 3 standard deviations of the value obtained with sera from healthy dengue and JE virus antibody-free Bangkok school children compared with the counts per minute obtained with a reference negative control serum of a dengue and JE virus antibody-free healthy American adult (cpm = 209). The mean and standard deviation of the P/N values obtained were 1.38 and 0.54. The positive cut-off was therefore defined as a P/N $\geq$3.1.

Assays of encephalitis patient and control sera. Figure 3 shows the P/N values obtained with acute- and convalescent-phase sera from 43 patients with clinical diagnoses of acute encephalitis. For each serum sample, the day of illness and the HAI titer code are shown. Overall, 19 of 20 patients with HAI-confirmed JE (groups IA and IB) had a positive ACRIA (P/N >3.0), whereas none of 10 flavivirus antibody-negative patients (group IC) was positive. Among 13 patients with equivocal HAI serological results (group ID), sera from 8 gave a positive result in the JE IgM ACRIA. The mean and standard deviation of the P/N ratios in HAI-confirmed cases were as follows: primary flavivirus infections, acute-phase sera, 14.8 $\pm$ 10.2; primary flavivirus infections, convalescent-phase sera, 34.9 $\pm$ 20.2; secondary flavivirus infection, acute-phase sera 10.1 $\pm$ 7.4; secondary flavivirus infections, convalescent-phase sera, 12.2 $\pm$ 6.9. Among 20 sera free of dengue and JE antibody from healthy children, 50 sera positive for JE antibody from healthy children, and 20 sera positive for JE antibody from healthy adults (total = 90), none had a positive assay (all P/N ratios $\leq$3.0).

Long-term follow-up sera. Late convalescent-phase serum specimens were obtained from seven cases who had positive JE IgM ACRIA antibodies in either the acute-phase or short-term convalescent sample (Table 1). Five of the seven patients showed persistently low levels of

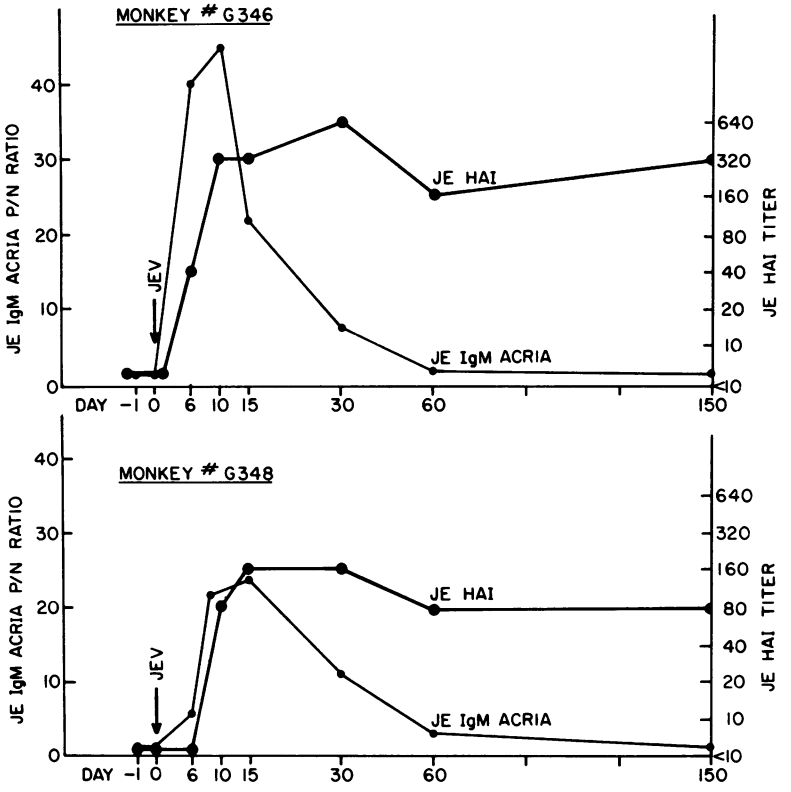

FIG. 2. Time course of appearance of JE IgM ACRIA and JE HAI activity in sera of monkeys infected with JE virus.

JE IgM ACRIA antibodies for periods longer than 9 months (mean follow-up period, 561 ± 173 days; mean P/N ratio of five positive cases, 8.6 ± 4.7).

Assays of sera from patients with dengue infections. Eighteen of 20 sera from children convalescent from acute dengue infections were found to have low-titered positive assays. Similarly, 3 of 10 sera from children who had converted from flavivirus seronegative to flavivirus seropositive sometime during the preceding 6 months were weakly positive.

Table 2 shows the P/N values obtained when sera from patients with acute encephalitis or hemorrhagic fever were assayed by the ACRIA method, using JE or dengue 2 virus as the test antigen. Reactions with dengue antigens were uniformly greater in hemorrhagic fever patients, whereas reactions with JE antigens were greater in the encephalitis patients.

Assays using RF-positive sera. Table 3 shows the P/N values obtained with serum specimens known to be RF positive. Five of 11 gave weak positive values; in four of these five sera, the P/N ratio with normal mouse brain antigen was 70% or more of the P/N when JE antigen was used.

Tabulated results. Results from all human specimens tested are summarized in Table 4.

DISCUSSION

Compared with currently used serological techniques for the diagnosis of JE infections, the JE IgM ACRIA has a number of advantages: (i) only small quantities (1 μl or less) of serum are required, (ii) serum pretreatment steps are eliminated, (iii) competition between IgG and IgM for antigenic sites is eliminated, and (iv) crude antigens can be used with excellent results.

We believe that the demonstration of a JE IgM ACRIA P/N ratio of greater than 3.0 in the blood of a patient with acute encephalitis is reasonable presumptive evidence that the acute illness is due to JE. Using this cut-off level, 80% of cases could be diagnosed at the time of hospital admission, and 95% of cases could be diagnosed within 2 weeks. However, biological false-positive reactions may occur when sera are tested from patients (i) who have had a prior encephalitic illness with JE, (ii) who have had a very recent infection with another flavivirus, or (iii) who have high serum titers of RF.

We found that low levels of JE IgM ACRIA antibodies may persist for 2 years or longer after

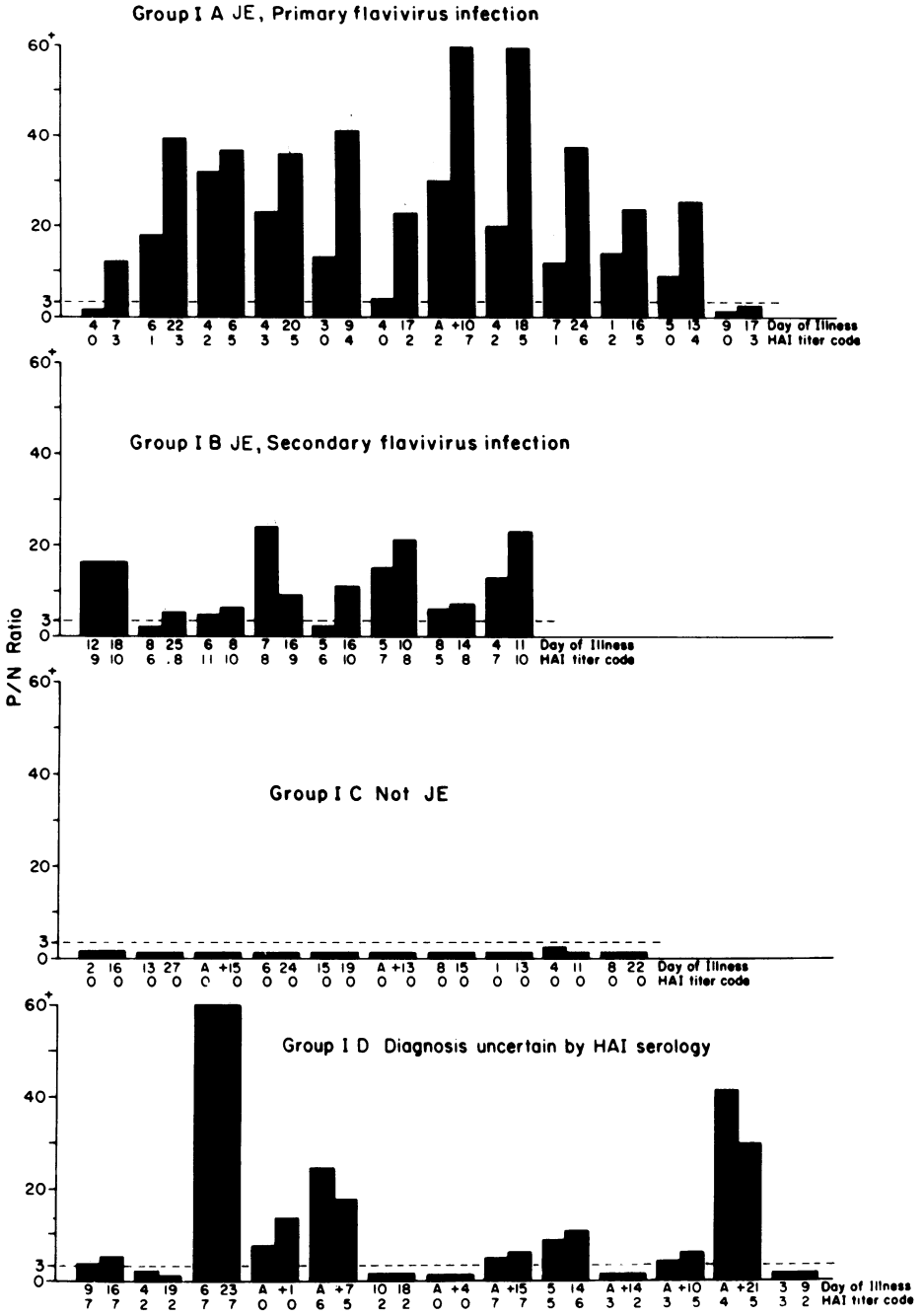

FIG. 3. JE IgM ACRIA activity in sera of patients with clinical diagnoses of acute encephalitis. The day of onset of illness was obtained from a standardized laboratory request slip as completed by the patient's physician; in instances in which only the calendar dates of the serum specimens are known, the day of onset of illness is recorded only as "A." The JE HAI titer code is the $\log_2$ (HAI titer/5) so that <1:10 = 0, 1:10 = 1, 1:20 = 2, 1:40 = 3, 1:80 = 4, 1:160 = 5, 1:320 = 6, 1:640 = 7, 1:1,280 = 8, 1:2,560 = 9, 1:5,120 = 10, and ≥1:10,240 = 11.

an acute encephalitic illness due to JE. Using sucrose density gradient fractionation to detect serum JE HAI antibodies, Edelman et al. (5) reported that among clinical cases of encephalitis due to JE, the duration of persistence of IgM anti-JE was directly related to the severity of illness. We do not have the clinical information to determine whether long-term persistence of

TABLE 1. JE IgM ACRIA activity in serum specimens obtained late (9 months to 2 years) after onset of encephalitis

Group	Days after initial serum specimen	JE HAI titer	JE IgM ACRIA (P/N)
IA	0	20	51.7
	14	640	126.9
	726	10	15.0
IA	0	10	44.1
	15	40	103.2
	621	10	1.8
IA	0	10	30.0
	8	80	102.0
	585	10	4.9
IA	0	10	95.1
	15	80	119.5
	349	80	8.4
IA	0	10	46.1
	6	160	109.1
	298	20	1.7
IB	0	80	9.6
	11	1,280	38.1
	739	1,280	3.6
ID	0	20	45.0
	—[a]	—	—
	608	2,560	11.3

[a] —, no convalescent-phase sera.

JE IgM ACRIA antibodies in five of the seven encephalitis patients we studied was related to the severity of their acute illness. However, none of 70 normal adults and children with serum HAI anti-JE antibodies had JE IgM ACRIA antibodies, suggesting that IgM anti-JE typically does not persist after subclinical JE infection. Taken together, the findings of Edelman et al. (5) and ourselves suggest that the duration of persistence of IgM JE antibodies may be directly related to the severity of the original infection across the entire spectrum of disease severity. Thus, in a patient with acute encephalitis without a history of prior overt encephalitis, a serum JE IgM ACRIA P/N ratio of greater than 3 is unlikely to be due to remote JE infection.

JE IgM ACRIA antibodies may also be found in patients acutely infected with flaviviruses other than JE. This is not likely to present a clinical problem in the differentiation between JE and other flaviviruses with clinical presentations dissimilar from JE. For example, dengue rarely if ever presents as acute encephalitis, and false-positive reactions due to dengue are unlikely to be encountered in specimens from patients with acute encephalitis. However, cross-reactive IgM dengue antibodies may persist at low levels for 6 months or longer and could cause diagnostic confusion in the case of a patient with acute encephalitis who had experienced a dengue infection sometime in the recent past. We did not test serum specimens from patients with encephalitis due to flaviviruses other than JE such as tick-borne encephalitis, West Nile, Murray Valley, or St. Louis encephalitis virus; it is likely that sera from patients infected with these viruses would also give biological false-positive reactions in the JE IgM ACRIA assay.

Although the IgG response to flaviviruses tends to be broadly reactive, the IgM response is reasonably specific. Using sera from JE- or dengue-infected patients, we demonstrated that the infecting flavivirus virus type (JE or dengue) could be identified by comparing the IgM ACRIA P/N ratios obtained with the homologous and heterologous flavivirus antigens. The same approach could probably be used to differentiate an infection with JE from one due to tick-borne encephalitis, West Nile, Murray Valley, or St. Louis encephalitis virus.

Although 19 of 20 HAI-confirmed cases had positive JE IgM ACRIA antibodies in either the acute- or convalescent-phase sera, we found one patient who was repeatedly negative despite a clear and repeatable seroconversion demonstrable by HAI on whole serum. The patient had a normal serum total IgM level as determined by quantitative enzyme-linked immunosorbent assay. 19S anti-JEV HAI activity could not be detected by sucrose density gradient fractionation analysis of either the acute- or convalescent-phase specimens in this case, whereas 7S activity was detected. Although it is possible that our assays simply failed to detect a low level of IgM anti-JEV, we believe that two other explanations are possible: (i) the seroconversion was an artifact or resulted from a specimen-handling error or (ii) the patient was infected by an unidentified flavivirus (other than dengue or JE virus) so that whole serum and 7S cross-reactions were detected, but the more specific JE IgM ACRIA assay failed to detect cross-reactive antibodies.

Sera from patients with high titers of RF may also give weak false-positive reactions when tested for JE IgM ACRIA antibodies; 5 of the 11 RF-positive specimens we tested gave P/N ratios greater than 3.0, but none was greater than 10.0. False-positive reactions due to RF were identified by substituting normal uninfected mouse brain for JE-infected mouse brain as the antigen; with true positive sera, the P/N ratio with normal mouse brain antigen fell to near unity, whereas sera with false-positives due to RF failed to show a marked decrease in the P/N ratio when normal mouse brain was substituted for JE-infected mouse brain.

The finding of a high JE IgM ACRIA P/N ratio (>10.0) in a serum specimen from a patient with

TABLE 2. Cross-reactions between flaviviruses detected by JE virus IgM ACRIA in convalescent-phase sera

Patient no.	Serum no.	Current infection	Previous flavivirus infection	P/N ratio		Ratio P/N JE/ P/N D2
				JE IgM ACRIA[a]	D2 IgM ACRIA[b]	
1	2503/79	Encephalitis	0	21.0	1.9	11.1
2	2424/79	Encephalitis	0	34.5	2.7	12.8
3	3036/79	Encephalitis	0	17.4	1.8	9.7
4	2303/79	Encephalitis	+	11.7	1.6	7.3
5	2403/79	Encephalitis	+	24.0	2.4	10.0
6	2513/79	Encephalitis	+	8.0	1.8	4.4
7	2700/79	Hemorrhagic fever	0	4.6	124.1	0.04
8	4329/79	Hemorrhagic fever	0	4.4	73.8	0.06
9	3824/79	Hemorrhagic fever	0	3.1	42.9	0.07
10	3382/79	Hemorrhagic fever	+	3.5	26.4	0.13
11	3796/79	Hemorrhagic fever	+	1.7	4.8	0.35
12	3850/79	Hemorrhagic fever	+	3.4	14.5	0.23

[a] $\bar{X}$NC = 852 cpm/well.
[b] $\bar{X}$NC = 187 cpm/well.

TABLE 3. RF-positive sera tested in the JE IgM ACRIA with infected mouse brains or normal mouse brains (NMB) as antigens

Identity	Series no.	RF titer	P/N value using:	
			JE antigen	NMB antigen
Negative control	1 (DB)		0.8	0.5
	2 (JB)		1.2	1.5
Positive control	1 (3032)		123.9	0.8
	2 (2503)		135.8	0.7
RF positive	1	1:40	3.5	4.0
	2	1:40	2.6	1.9
	3	1:40	1.3	0.6
	4	1:40	3.4	1.0
	5	1:80	4.1	2.9
	6	1:80	5.0	3.6
	7	1:80	3.0	1.4
	8	1:160	2.5	0.9
	9	1:160	3.0	2.2
	10	1:320	6.6	5.0
	11	1:640	3.0	2.2

TABLE 4. Results of JE IgM ACRIA on all human serum specimens tested

Group	Timing of sera	No. tested	No. of cases with P/N greater than:		
			3.0	5.0	10.0
Healthy antibody-negative school children		20	0	0	0
IA, encephalitis, 1° JE	Acute	12	10	9	8
	Convalescent	12	11	11	11
IB, encephalitis, 2° JE	Acute	8	6	6	4
	Convalescent	8	8	7	4
IC, encephalitis, not JE	Acute	10	0	0	0
	Convalescent	10	0	0	0
ID, encephalitis, ? JE	Acute	13	8	5	3
	Convalescent	13	8	8	5
II, healthy adults		20	0	0	0
IIIA, healthy children		50	0	0	0
IIIB, healthy children		10	3	0	0
IVA, convalescent dengue, 1°		10	10	7	1
IVB, convalescent dengue, 2°		10	8	2	0
V, rheumatoid factor		11	5	1	0

acute encephalitis in Thailand is virtually diagnostic of an acute infection with JE virus. Most JE patients have JE IgM ACRIA antibodies of this level in serum drawn at the time of hospital admission and can rapidly be diagnosed as JE with a high degree of accuracy. Patients with negative (P/N $\leq$3.0) or weak positive (P/N >3.0 to 10.0) test results should have an additional specimen drawn and tested, as serum JE IgM ACRIA antibodies continue to rise after hospital admission. Specimens with weak positive results should be retested with both dengue antigen and normal mouse brain control antigen to identify biological false-positive reactions due, respectively, to IgM antibodies and RF. If both these types of biological false-positives are eliminated, sera with JE IgM ACRIA P/N ratios of >3.0 can be considered diagnostic of JE virus infection.

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