Attempted Type Specific Diagnosis of Dengue Virus Infection by the Indirect Fluorescent Antibody Method Directed at Differentiating IgM and IgG Responses[1] (37097)

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The need for paired acute and convalescent phase sera for diagnostic purposes in virology has long been recognized. Along these lines, some important problems are: (a) getting an early enough acute phase serum, (b) obtaining only a convalescent phase serum perhaps none at all, (c) choice of most suitable test method(s) to use with likely small quantities of specimen(s) obtained and (d) perhaps most important of all, furnishing a diagnostically useful result to the attending physician at the time he needs it. This latter requirement is incompatable with awaiting a convalescent phase serum. Furthermore, unless the physician's early needs are met, his subsequent lack of cooperation further complicates obtaining the needed sera from the next patient.

The solution in so far as serology is concerned apparently lies in the detection of early specific antibody in the IgM globulin fraction and distinguishing this from that of any preexisting antibody in the IgG globulin which may also react with the antigen used. Since fractionation procedures to separate IgG or selectively inactivate IgM are time consuming or impractical in large scale routine diagnostic work, the indirect fluorescent antibody technique employing highly specific conjugates against human IgM and human IgG appears to be most desirable. Diagnostic use of such a procedure in virology was introduced by Brown and his associates in 1968 (1, 2) for mumps, but to our knowledge this has not been applied in diagnostic arbovirology prior to the studies reported here.

The major interest in this laboratory is in the area of the arboviruses and currently in the dengue (DEN) subgroup of group B most specifically, therefore these viruses were employed. Another reason for selecting group B and the DEN subgroup relates to the lack of type specificity, using current serological tests, of sera of persons having a second or subsequent infection with a group B virus. Furthermore, group B infections of major importance occur frequently in tropical areas where from 4 to 8 such related viruses may be active. In such cases of repeated group B infections the detectable antibody to the group antigen(s) is in the IgG fraction, appears early and in high titer (3). At about the same time or even later the type specific antibody to the current, infecting virus type (a primary type response) is expected to be found in much lower titer in the IgM fraction. Since the experimental results obtained in the present study appear to have limited positive value, this report is brief. Access to well-documented, fresh, human sera limited the scope of the study.

Materials and Methods. Virus strains.

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TABLE I. Results of Fluorescent Antibody Tests on Sera from a DEN-2 Inoculated Rhesus Monkey Tested Against DEN-2 and DEN-4 Antigen(s).

Serum no.	Day post-inoculation	Indirect FAS test results			
		IgM conjugate[a]		IgG conjugate[a]	
		DEN-2	DEN-4	DEN-2	DEN-4
1	3	—[b]	—	—	—
2	4	—	—	—	—
3	5	++	—	—	—
4	9	+	+	++	+
5	30	—	—	+++	++
Control neg.[c]		—	—	—	—
Control DEN-2[d]		ND[f]	ND	++++	ND
Control DEN-4[e]		ND	ND	ND	+++

[a] Uninfected LLC-MK_2 cells were negative with all the sera and conjugates used.

[b] Negative staining, —; specific staining: graded ±–4+ in intensity; ± not always reproducible on repeat testing.

[c] Normal monkey serum with no HI titer to any dengue virus type.

[d] DEN-2 monkey antiserum.

[e] DEN-4 human antiserum.

[f] Not done.

DEN-1, Hawaiian strain in mouse passage (mp) 23; DEN-2, New Guinea (NG) "C" in mp 23; and DEN-4, H241 in mp 21 were used for most of the work. Stocks were frozen in ampoules as 20% suckling mouse brain (smb) suspensions at about —65°. For some experiments with DEN-2, Vero cell passages were made from the smb stock, and for DEN-4, passages were similarly made in LLC-MK_2 cells. From the cell cultures the undiluted fluid harvest was frozen for stock.

Cell cultures. Vero, secondary hamster kidney, and LLC-MK_2 cells were all tested, the latter two types both exhibiting brightly staining antigens. All final tests reported here, however, were with the LLC-MK_2 cell line. These cells were grown at 37° on coverslips in 16 × 125 mm screw-capped tubes until a complete monolayer formed. Growth medium was Eagle's basal medium (EBM; General Biochemical, Inc.) containing 8% heat-inactivated newborn calf serum (General Biochemical, Inc.), 0.04% sodium bicarbonate and final concentrations of penicillin at 100 units/ml, streptomycin at 40 μg/ml and polymyxin B at 10 units/ml. Maintenance medium for cell cultures was modified from the above to contain concentrations of 5% serum and 0.12% sodium bicarbonate. Virus (0.1 ml) diluted 10^{-1} from the frozen stock was placed on each drained coverslip and permitted to adsorb for 1 hr at 37°. The coverslips were drained and then replaced in the tubes with a change of maintenance medium. Medium used for virus inoculation of cells was similar to regular maintenance medium except 2% agamma calf serum (North American Biologicals, Inc.) was substituted for the newborn serum. Incubation was continued at 37° for 48 hr for DEN-1 and DEN-2 and 72 hr for DEN-4. Uninoculated monolayers of cells on coverslips were maintained as controls. CPE was not manifest at this time. Fixation in acetone was carried out as described by Atchison *et al.* (4). After drying, the coverslips were stained immediately or stored up to 2 wk at 4° and/or —20° for longer periods.

Conjugated sera. Highly specific goat anti-human IgM and IgG (heavy chain) sera labeled with fluorescein isothiocyanate were purchased from Kallestad Labs, Inc., Minneapolis, MN and used in a 1:4 dilution in 0.01 Sorensen's sodium phosphate buffer with 0.85% NaCl (SPBS). These sera were checked for specificity and activity against human and monkey sera by immunodiffusion (Ouchterlony) and immunoelectrophoresis and found entirely satisfactory.

Fluorescent antibody staining and examination. The test procedures, including all the appropriate controls for specificity for the indirect type of staining, were essentially those of Coons, Ledue and Connolly (5) and of Riggs (6). The microscopy equipment used was that described previously by Atchison *et al.* (4). Staining of the cytoplasm of dengue-infected cells in conjunction with appropriate hyperimmune monkey dengue antisera or late convalescent phase human sera was an intense 3+ or 4+. In a very few other cells nonspecific fluorescence sometimes occurred, was very faint and of a distinctly different color.

Results. Serial bleedings were available through Day 30 following inoculation from a rhesus monkey, previously free from detect-

TABLE II. Results of Fluorescent Antibody Test on Serial Sera from Volunteers Injected with DEN-1 Hawaiian Virus.

Volunteer	Days post-inoculation	Days after onset	FA reaction[a] IgM conjugate DEN-1	IgM conjugate DEN-2	IgM conjugate DEN-4	IgG conjugate DEN-1	IgG conjugate DEN-2	IgG conjugate DEN-4
BR	0		—	ND	ND	—	ND	ND
	10	2	—	ND	ND	—	ND	ND
	15	7	+++	++	++	++	++	—
	21	13	+	+	++	—	++	±
FO	0		—	ND	ND	—	ND	ND
	10	2	—	ND	ND	—	ND	ND
	15	7	++	+	—	—	+	—
	21	13	—	+	—	++	++	++
	29	21	—	—	—	+++	+++	++
SNY	0		—	ND	ND	—	ND	ND
	10	3	—	—	—	—	—	—
	15	8	++	—	±	+	+	±
	21	14	—	—	—	++	+++	++
G.M.	0		—	ND	ND	—	ND	ND
	10	0	—	ND	ND	—	ND	ND
	15	5	+	—	+	—	+	—
	21	11	++	+	++	++	++	++
	29	19	—	+	+	+++	++++	++
FUL	0		—	ND	ND	—	ND	ND
	10		—	ND	ND	—	ND	ND
	15		++	++	±	++	+	—
	21	3	±	+	±	+++	±	—
Control neg			—	±	—	—	±	—
Control DEN-1			ND	ND	ND	+++	ND	ND
Control DEN-2			ND	ND	ND	ND	+++	ND
Control DEN-4			ND	ND	ND	ND	ND	++++

[a] See footnotes *b* and *f* Table I.

able group B hemagglutination inhibiting (HI) antibody, inoculated here 8 yr before with one injection subcutaneously of DEN-2 (NG "C" smb P.24). It had developed viremia 3 days after inoculation and later neutralizing (N), but not complement fixing (CF) antibodies. Results of the fluorescent antibody (FA) tests with DEN-2 (homologous) and DEN-4 are shown in Table I. Moderate (1+ to 2+) specific staining was observed with Day 5 and 9 sera using the IgM conjugate, and first on Day 9 using the IgG conjugate. On Day 5 the IgM response appeared quite specific, and as yet there was no detected IgG response.

Through the kindness of Dr. Charles Wisseman we obtained early serial sera from 6 American volunteers he had used to titrate DEN-1 virus in 1959. All but the one receiving the highest dilution was reported to have developed classical dengue fever and a neutralizing antibody response. Sera were frozen when received. On tests for CF antibody here, none reacted, due possibly to the fact that the last serum was either drawn 21 days after inoculation (3 to 14 days after onset of disease), or 29 days after inoculation (19 to 21 days after onset). Results of the FA tests with DEN-1, -2 and -4 for the five infected are shown in Table II. Results were negative for all sera of the one non-infected volunteer.

It will be noted that reactions with the IgM conjugate during these primary infections were not noted in all the early sera, and when observed were usually at a level

TABLE III. The Results of Fluorescent Antibody Tests on Paired Thai Sera from Probable Primary Dengue Infections.

Child no. and serum no.		IgM conjugate		IgG conjugate	
		DEN-2	DEN-4	DEN-2	DEN-4
No. 14	1	—[d]	—	—	—
	2	—	+	—	±
No. 123	1	—	—	+	—
	2	—	+++	—	—
No. 209	1	—	—	—	—
	2	—	—	—	++
Control neg.[a]		—	—	—	—
Control DEN-2[b]		ND[e]	ND	+++	ND
Control DEN-4[c]		ND	ND	ND	++++

[a] Human serum with no HI titer to any dengue type.

[b] Dengue human antiserum with HI titer to DEN-2 virus 1:2048.

[c] Dengue human antiserum with HI titer to DEN-4 virus 1:2048.

[d] No staining.

[e] Not done.

graded less intense than those of IgG. Furthermore, there was little evidence of discriminating viral type specificity by the IgM antibody.

Three paired sera (approx 1 mo interval) from a survey of normal Thai children collected from the island of Koh Samui near Thailand in May and again June of 1968 were sent through the kindness of Lt. Col. Thomas J. Smith, MC, USA. They were received in a lyophilized state. In tests made in Bangkok the second sera had converted by HI test to DEN-4 in the interval, but no clinical disease was noted. From the low HI titers (1:80 to 1:640) and the relative lack of cross reactions shown to the other 3 dengue types these were assumed to be primary group B infections due to DEN-4 virus. CF tests performed here showed a 1:16 antibody titer to DEN-4 antigen in only one of the 3 late sera while all others, early and late failed to react at 1:8 with any of the 4 DEN antigens.

The results of FA testing are shown in Table III. Two of the late sera reacted specifically to DEN-4 in the presence of the IgM conjugate but a questionable reaction with DEN-4 virus and IgG occurred also in one of these. The third child probably converted soon after the first serum was drawn for the second serum showed only a clear-cut IgG reaction, also specific for DEN-4.

Other sera, some paired and some single acute phase from secondary type dengue infections from Thailand, most of them diagnosed clinically as hemorrhagic fever, were collected by one of us (WMH) in 1958 and by Dr. Ethel R. Nelson in 1960 and were stored here frozen at about —30° soon after shipment in dry ice. All had been previously shown to be group B virus infections by serology performed here, and DEN viruses had been isolated and typed from four of the patients. Results of the earlier CF tests and virus typing and the present FA tests are shown in Table IV.

As expected, all that reacted (excepting YPS on Day 1 after onset) reacted as rapidly and to higher titer with the IgG conjugate than with the IgM. Those that did react to the IgM conjugate usually did so only, or to a greater degree in the second serum; this was always during the second week after onset.

Of the four patients from whom viruses were isolated, three type 2 and one type 4, in one (TH-31) type specific FA reactions occurred with the IgM conjugates at 12 days but not at 3 days. In another (BH-28) not at all at 2 or 8 days, in another (TH-33) not at 2 days, but in the last (YKS), at 5 days. Since these were secondary type responses, as shown by CF, the simultaneous IgG reactions were not unexpected. All other IgM reactions were with DEN-2, not DEN-4, but without isolations there was no way to determine the specificity. All controls in these tests were as expected as in all other reported tests.

Discussion. It is recognized that detection of antibodies in the IgM or IgG fractions of gamma globulin by the methods employed or referred to do not necessarily reflect the actual quantity of such antibody, or the time when formed, but instead may reflect the avidity or activity demonstrated in the test

TABLE IV. Results of Fluorescent Antibody and Complement Fixation Tests on Patients' Sera from Bangkok 1958 and 1960.

Patient	Days after onset	CF titers[a]				FA reactions[b]				Virus isolate
						IgM		IgG		
		DEN-1	DEN-2	DEN-3	DEN-4	DEN-2	DEN-4	DEN-2	DEN-4	
TH-31	3	<4	<4	<4	<4	−	−	+	−	DEN-2
	12	32	128	8	512	+	−	++++	++++	−
WHH	2	ND	<4	ND	ND	±	−	−	+	−
	9	ND	>128	ND	ND	+	−	++	+++	−
BH-28	2	ND	<4	ND	ND	−	−	−	−	DEN-4
	8	ND	>128	ND	ND	−	−	+++	+	−
LKL	3	32	32	ND	ND	+	−	+++	+	−
	10	>128	>128	ND	ND	ND	−	+++	+	−
NT	1	8	8	ND	ND	+	−	+++	++	−
	9	>128	>128	ND	ND	+	−	++++	++	−
OU	4	16	32	ND	ND	±	−	+++	−	−
	14	>128	>128	ND	ND	+	−	++++	−	−
TH-33	2	<4	<4	<4	<4	−	−	++	±	DEN-2
	16	64	512	64	>1024	QNS	QNS	QNS	QNS	−
YKS	5	16	16	4	16	++	±	+++	++	DEN-2
	17	1024	1024	64	2048	QNS	QNS	QNS	QNS	−
TP	2	<4	<4	<4	<4	±	−	++	−	−
	12	1024	1024	1024	512	QNS	QNS	QNS	QNS	−
Cont. Neg.						−	−	−	−	
Cont. DEN-2						ND	ND	++++	ND	
Cont. Den-4						ND	ND	ND	++++	

[a] Tests performed between 1956 and 1960 using the method described by Hammon and Sather (7).

[b] See footnotes *b* and *f*, Table I.

used. Since different types of serological tests may reflect different avidities and little is known about fluorescent antibody tests in dengue infection, the occasional, apparent inconsistencies from the expected pattern may result from such causes. It had been hoped that it would be possible to demonstrate clear-cut, early, type specific indirect FA reactions in a system using goat anti-human IgM (heavy chain) in primary dengue infections, possibly even without the need of an anti-human IgG serum. By having fixed coverslips (stored frozen) with each type of DEN virus-infected cell monolayers, a type specific diagnosis of dengue could thus be made in a few hours after an acute phase serum was received. With a similar frozen store of coverslips with Japanese encephalitis or other group B virus-infected cell monolayers, other group B virus infections could be similarly, quickly diagnosed. It appears from the results obtained that occasionally such may be possible, but that one should certainly use anti-IgG in addition. Unfortunately the results obtained here indicate a low sensitivity for the IgM antibody and possibly for this reason a very limited period of time during which the IgM antibody can be detected. This however, in view of the well-recognized lability of IgM may be due entirely to the age and condition of storage of the sera we had available or were able to obtain. Information on the manner of storage or how many times each serum was frozen and thawed was not available for many of the sera. Our sera had all been frozen and thawed at least once and some several times and had been stored for 11 to 13 yr at −25 to −30°. All the others had been previously tested and also had been stored for a number of years. Therefore, it is quite possible that fresh sera would contain

much larger quantities of detectable IgM antibody and if so it would give stronger reactions and be detectable over a longer period of time than shown here. The most discouraging aspect of the results was the apparent lack of type specific response to a known, single dengue infection (DEN-1) shown by some of the human volunteers, *i.e.*, BR, GM and FUL (Table II).

In respect to secondary type dengue infections in which no other serologic method now employed is able to determine the type of the current infection in the group B arboviruses, this test offers some suggestive promise of help. This prospect should be tested using freshly drawn serum or that stored properly for these purposes so that the sensitivity of the test could be increased perhaps. It would appear highly improbable that in a secondary type of infection of a degree adequate to produce a typical clinical disease manifestation and immunity to subsequent homologous reinfection there would fail to be stimulated primary, type specific IgM antibody to this first encounter with a unique type specific antigen. If this does not occur one is forced to consider the possibility that each of the dengue viruses and perhaps all in the same subgroup of group B contain all antigens possessed by any of the others (all group antigens, none type specific) and that antigenic differences between viruses are quantitative only. This would seem highly improbable on the basis of all present knowledge and concept.

It is hoped that those carrying out diagnostic tests in areas of endemic or epidemic mixed grouped B arbovirus infections will give this type of test a thorough trial with freshly collected, well-preserved serial sera from patients.

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