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A Specific Complement-Fixation Test for Human Hepatitis A Employing CR326 Virus Antigen. Diagnosis and Epidemiology (38669)

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Studies of human hepatitis A (infectious hepatitis) have been hampered by the lack of a simple assay for antibody against hepatitis A virus. Such a test has been developed as the present report will show.

Historically, Deinhardt *et al.* (1) first presented evidence for propagation of hepatitis A virus in *Saguinus nigricollis* and *Saguinus oedipus* marmosets. Provost *et al.* (2) in our laboratories developed an assay for neutralizing antibody against hepatitis A virus in *S. mystax* marmosets and provided definitive proof of etiologic relationship between human hepatitis A isolate CR326 and human hepatitis A. The serum neutralization findings were confirmed by Holmes *et al.* (3). Using another approach, Feinstone *et al.* (4) later demonstrated a 27 nm diameter particle in the stool filtrate of a case of hepatitis A and the development of an antibody against it that was detected by immune electron microscopy.

The present report describes the development of a specific complement-fixation (CF) test for human hepatitis A antibody employing, as antigen, liver extract of marmosets infected with the CR326 strain (2, 5) of human hepatitis A virus. Data relating to the development of CF antibody against the virus in hepatitis A cases are presented and discussed.

Materials and Methods. Virus. The CR326 strain (5) of human hepatitis A virus isolated in marmosets from a case of hepatitis A in Costa Rica was used to prepare CF antigen. *Preparation of CF antigen.* The hepatitis A CF antigen preparations were made from livers of *S. mystax* marmosets infected intravenously with CR326 virus (2, 5). The livers were collected from the animals at a time when the serum enzyme values (SGOT and SICD) were elevated. The control CF antigens were made from livers of uninfected marmosets. All livers were perfused thoroughly with phosphate buffered saline (PBS), pH 7.2, minced with a scissors, and ground in a mortar with sterile alundum to give a 10% suspension by weight in PBS. The antigens consisted of the supernatant fluids obtained after low speed clarification. Both viral and control antigens were heated at 56° for 2 hr prior to use in the CF tests. *Hepatitis A complement-fixation (CF) tests.* Grid titrations were performed using serial dilutions of human hepatitis A convalescent serum and serial dilutions of antigen. The CF antigen titers were low and it was usually necessary to employ the infected liver preparation at a 1:2 dilution (2 units of antigen) in the tests for hepatitis A antibody. The normal control CF antigen was used at the same dilution. Two and one half full units of guinea pig complement were employed in the tests. Incubation with complement was overnight at 4°. The hemolytic system consisted of a 1% suspension of sensitized sheep red blood cells. Antibody titers were expressed as the highest initial dilution of serum giving 50% or greater fixation of complement. *Hepatitis B CF tests.* Tests for the surface antigen (HB_sAg, Australia antigen) were performed according to Purcell *et al.* (6).

Patients' sera. Natural hepatitis A and B cases in Costa Rica. These were cases of hepatitis A or B that occurred among persons who resided in the province of Alajuela, Costa Rica and who were subjects in the large-scale epidemiologic studies that were conducted there (2, 5, 7, 8). They bear eight digit identification (e.g., 206-033-02). Onset of illness was taken as the first day of clinically detectable disease. All cases were con-

firmed by standard clinical laboratory tests. Some of the same sera were assayed for neutralizing antibody in the marmoset test (2). Some of the neutralization findings were reported previously (2). *Coded specimens from persons experimentally infected with hepatitis A virus.* Sera from the three subjects listed in Table II had received MS-1 hepatitis A virus at Willowbrook State School in studies carried out by Dr. Saul Krugman and his associates (9, 10). The sera were kindly furnished to us under code by Dr. Krugman and the code was not broken until after the tests were completed. *Normal sera.* The "normal" sera were collected in routine bleedings from 22 young adult employees of these laboratories (West Point).

Results. Naturally occurring hepatitis A cases in Costa Rica. The findings in eight cases of hepatitis A that occurred with onset during 1967–1968 are presented in Table I. The serum neutralizing antibody values of sera from six of the cases are as shown. In the neutralization tests, a value of 50 or greater was considered to be positive and a difference in the values of 50 or greater in tests of paired sera was considered to be significant (2). The findings in the first three cases in the table are also presented graphically in Figs. 1, 2 and 3. All the patients developed CF antibody against hepatitis A antigen shortly following onset of illness; it persisted undiminished for as long as 185 days (the longest period tested). The development of hepatitis A CF antibody correlated with the development of neutralizing antibody. Hepatitis A neutralizing antibody was detected 2 days after disease onset in patient 204-538-11 and CF antibody was present 9 days after onset in patient 204-538-11. The patients' sera frequently (first four cases in the table) became highly anticomplementary around the time of onset of illness and this diminished rapidly thereafter. All the patients' sera reacted, to some extent, with normal liver control antigen and the serum titer increase against normal antigen seen in the first four patients in the table was likely due to the anticomplementary activity. The increase in titer against the hepatitis A antigen, unlike that against normal liver antigen, was greater, was always sustained, and therefore, provided a basis for confidence in the reliability and specificity of the assay for hepatitis A antibody. Further, the hepatitis A antibody was not removed by absorption, three times, with acetone-dried normal liver antigen. The elevated titer against normal liver antigen was sustained in only one patient (068-330-08) and the titer was at least 16-fold lower than for hepatitis A antibody.

Experimentally infected hepatitis A cases. The specimens from the three cases of hepatitis A shown in Table II were sent to us under code by Dr. Saul Krugman and were tested blindly. The findings, compiled after the code was broken, are as shown. These sera tended to be highly anticomplementary. The findings for hepatitis A CF antibody in these blind studies fully confirmed the results of tests of sera from hepatitis A cases shown in Table I.

Hepatitis B cases. The findings for hepatitis A CF antibody in four cases of hepatitis B, diagnosed by demonstrating HB_sAg in the sera, are shown in Table III. None of the persons showed hepatitis A antibody development as a result of hepatitis B infection. At least two of the patients (202-039-03 and 206-343-08) and possibly a third (202-039-05) had hepatitis A CF antibody resulting from previous hepatitis A infection. Unlike some hepatitis A cases, these patients did not show the marked anticomplementary activity in the serum samples taken around the time of onset of illness.

Hepatitis A and B. Family epidemiology. Table IV summarizes the occurrence of hepatitis A and B in a Costa Rican family followed from 3/2/67 through 5/30/67. Case 06, age 9, thought to be the hepatitis A index case, showed clinical onset on 3/2/67. It was, however, a case of hepatitis B in a subject who had previously had hepatitis A, as revealed by the hepatitis A antibody and hepatitis B antigen test results. The true index case of hepatitis A was subject 09, age 4, with onset 4/20/67. A second case of hepatitis A occurred in subject 08, age 5, with onset on 5/2/67. Family members 05 and 07 (age 10 and 7

TABLE I. HEPATITIS A COMPLEMENT FIXATION AND NEUTRALIZATION TEST RESULTS, HUMAN HEPATITIS A CASES, COSTA RICA.

Case no. (age, yrs)	Time of specimen (days)	CF antibody titers			Hepatitis A neutralizing antibody value
		Hepatitis A virus antigen	Normal liver antigen	AC[a]	
207-056-08	−57	20	20	10	
(5 yr male)	−28	10	20	5	
	0	20	40	5	
(Fig. 1)	+3	320	320	160	
	+91	320	40	<5	
	+185	640	40	5	
068-330-08	−48	5	10	5	−14[b]
(7 yr male)	−35	10	10	5	
	+2	40	80	40	
(Fig. 2)	+9	160	80	5	
	+31	640	80	5	
	+122	1280	40	5	
	+122 +183				89[b]
204-538-11	−20				0[b]
(10 yr male)	−12	20	40	5	
	−5				0[b]
(Fig. 3)	+2	80	80	40	51[b]
	+9	320	40	5	
	+90	640	40	10	
	+90 +178				78[b]
206-033-02	−37				−26[c]
(11 yr male)	−21	40	80	40	
	+36	2560	320	160	70[c]
	+102	2560	40	10	63[c]
203-035-09	−25				20[c]
(8 yr male)	+3	40	80	20	
	+39				78[c]
	+100	640	40	5	
702-207-05	−28				10[c]
(4 yr female)	+14	5	10	5	
	+45	640	20	5	
	+45 +112				91[c]
202-209-07	−28	10	10	5	18[c]
(10 yr female)	+29 +91				89[c]
	+185	640	10	<5	
206-343-07[d]	−18	5	10	5	
(8 yr female)	+9	80	20	<5	
	+183	80	20	5	

[a] Anticomplementary activity.
[b] Not previously published.
[c] Previously published (2).
[d] This patient was an HB_sAg carrier. All sera were positive for the HB_sAg when tested by CF (6).

yr) appeared to be hepatitis A immunes because of the high titer of hepatitis A antibody in their sera. Family member 10 (age 2 yr) appeared to be a susceptible because of lack of antibody. The older family members (01, 02, 03 and 04, ages 40, 37, 14 and 13 yr, respectively) were indeterminant because of the high level of reaction with normal liver antigen. It is probable that hepatitis A CF antibody diminishes with time after infection. *Normal human sera.* As seen in Table V, only two of 22 young

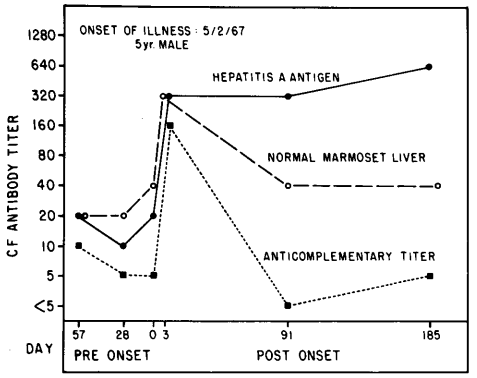

FIG. 1. Hepatitis A CF Titers, Costa Rica Hepatitis A Case 207-056-08.

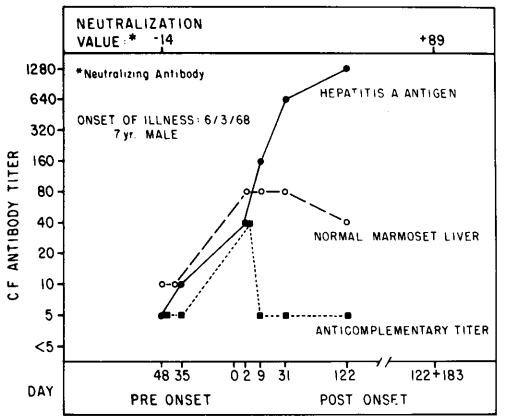

FIG. 2. Hepatitis A CF antibody titers and neutralizing antibody values, Costa Rica hepatitis A case 068-330-08.

adult workers (1, 2) in these laboratories (West Point) had detectable hepatitis A CF antibody in their sera. Both positives had a titer of 1:160. One positive subject was from Puerto Rico and the other had worked as a technician in a blood bank prior to present employment.

Titrations for hepatitis A CF antigen in marmoset liver suspensions. A total of 23 hepatitis A and three normal marmoset liver antigens were prepared and tested by CF using 4 units of hepatitis A antibody in human convalescent serum. The hepatitis A antigens ranged in titer from 1:2 to 1:16, most often 1:2 and 1:4. Only those titering 1:4 or greater were used to test antisera. None of three normal marmoset liver preparations contained detectable hepatitis A CF antigen.

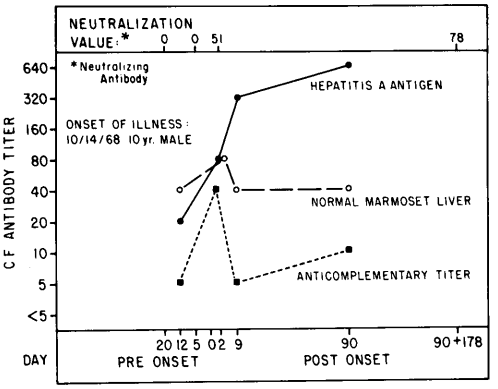

FIG. 3. Hepatitis A CF antibody titers and neutralizing antibody values, Costa Rica hepatitis A case 204-538-11.

TABLE II. HEPATITIS A COMPLEMENT-FIXATION TITERS, CODED SPECIMENS, HEPATITIS A CASES, IN EXPOSED INDIVIDUALS (BLIND STUDY, CASES OF DR. SAUL KRUGMAN).

Case no.	Day after exposure	SGOT	CF antibody titers: Hepatitis A virus antigen	CF antibody titers: Normal liver antigen	CF antibody titers: AC[a]
1	10		80	80	80
	35	Rise[b]			
	59		160	40	20
	71		640	40	40
	156		640	80	40
2	13		40	40	40
	30		10	10	10
	37	Rise			
	70		160	40	40
	3¾ yr		160	<10	<10
3	13		20	20	20
	30		40	80	40
	37	Rise			
	63		320	80	40

[a] Anticomplementary activity.

[b] First significant elevation in serum SGOT level.

Discussion. For decades, workers have sought simple and practical test procedures for unraveling the enigmas of human hepatitis A. The reliable propagation (1, 2, 5) of hepatitis A virus in marmosets opened the door to development of new technology for *in vitro* assay of hepatitis A antigen and antibody. The present report describes the first such assay by the CF method employ-

TABLE III. HEPATITIS A COMPLEMENT-FIXATION TEST RESULTS, HUMAN HEPATITIS B CASES, COSTA RICA.

Case no. (age, yrs)	Time of specimen (days)	CF antibody titers: Hepatitis A virus antigen	CF antibody titers: Normal liver antigen	CF antibody titers: AC[a]	Hepatitis A neutralizing antibody value	Hepatitis B antigen
202-039-01	−108	20	20	10		0
(36 yr male)	+10	5	10	<5		+
	+149	20	20	10		0
202-039-03	−27	80	10	5		0
(14 yr male)	+7	80	10	5		+
	+192	40	10	<5		0
202-039-05	−18				50[b]	
(10 yr female)	−4	40	10	5		+
	+8	40	20	5		+
	+190	40	10	5	67[b]	0
206-343-08	+6	320	40	10		+
(6 yr female)	+119	160	20	5		+

[a] Anticomplementary activity.
[b] Previously published (2).

TABLE IV. FAMILY OCCURRENCE, HEPATITIS A AND B, COSTA RICA.

Family 207-056: Member Code	Age (yr)	Sex	Date onset of hepatitis (1967)	Date serum specimens (1967)	CF antibody titers: Hepatitis A virus antigen	CF antibody titers: Normal liver antigen	CF antibody titers: AC[a]	Hepatitis B antigen
06[b]	9	M	3/2	3/7	320	20	5	+
				6/13	320	20	5	+
09[b]	4	F	4/20	3/6	20	40	10	0
				11/6	640	20	5	0
08[b]	5	M	5/2	3/6	20	40	<5	0
				8/4	320	40	<5	0
01	40	M	Not ill	3/6	80	40	<5	0
				5/30	40	40	<5	0
02	37	F	" "	3/6	40	40	20	0
				5/30	40	40	20	0
03	14	F	" "	3/6	160	40	10	0
				6/1	40	40	10	0
04	13	M	" "	3/13	80	20	<10	0
				5/30	80	80	10	0
05	10	F	" "	3/6	160	10	5	0
				5/30	160	20	10	0
07	7	M	" "	3/6	160	20	5	0
				5/30	160	80	10	0
10	2	M	" "	3/6	10	20	<5	0
				5/30	10	20	<5	0

[a] Anticomplementary activity.
[b] Case 06 was icteric with enzyme elevations. Case 09 was clinically anicteric hepatitis with enzyme elevations. Case 08 was subclinical hepatitis but with enzyme elevations.

TABLE V. HEPATITIS A COMPLEMENT-FIXING ANTIBODY TITERS—22 NORMAL PERSONS, WEST POINT, PENNSYLVANIA.

Serum no.	CF antibody titers[a]		
	Hepatitis A virus antigen	Normal liver antigen	AC
1	160	40	10
2	160	40	10
3	10	40	10
4	10	40	5
5	10	20	10
6	10	10	10
7	10	5	5
8	10	5	5
9	5	20	5
10	5	10	5
11	5	10	5
12	5	10	5
13	5	10	5
14	5	5	5
15	5	5	5
16	5	5	<5
17	<5	5	<5
18	<5	5	<5
19	<5	5	<5
20	<5	5	<5
21	<5	<5	<5
22	<5	<5	<5

[a] None of the sera contained HB_sAg.

ing, as antigen, CR326 strain human hepatitis A virus in liver of an infected marmoset. These studies, while only a beginning, present information on etiologic diagnosis of hepatitis A and data that may be of importance in describing the epidemiology of the disease.

In the study, it was shown that all of seven cases of suspect hepatitis A and one case of hepatitis A that had been misdiagnosed as hepatitis B because of HB_sAg antigenemia developed CF antibody against hepatitis A virus in the progress of their infections. The development of hepatitis A CF antibody was correlated with the development of neutralizing antibody. The precise time for first appearance of CF antibody was obscured by the development of anticomplementary activity in the acute phase sera but CF antibody, like neutralizing antibody, appeared to be present shortly after onset of clinical illness. In most cases, the maximal CF antibody titer was reached within the first month after onset of the acute disease. Titers persisted for as long as 3.75 yr, the longest period tested. The validity of the CF test results was supported by the consistent findings with coded sera sent to us for blind testing by Dr. Saul Krugman. Persons with hepatitis B did not develop CF antibody against hepatitis A antigen. One patient developed hepatitis A infection during the time that he was carrying HB_sAg (hepatitis B surface antigen) in his blood.

It was possible to describe the epidemiology of hepatitis in a family outbreak in Costa Rica by use of the CF test. Two of the family members (08 and 09, aged 5 and 4 yr) developed hepatitis A during the course of the study. Member 06, aged 9 yr, had previously had hepatitis A based on presence of antibody, but contracted hepatitis B more recently and this was evidently the cause for the present illness. Thus, it appears that both hepatitis A and B were occurring in the family during the same time period. The other family members did not develop hepatitis during the time period of the study. Members 05 and 07, aged 10 and 7 yr, had relatively high hepatitis A antibody levels and were probably immunes. Members 01, 02, 03 and 04 were of older age (40, 37, 14 and 13 yr, respectively) and might have lost their CF antibody had they had hepatitis A earlier. Member 10, age 2 yr, was without hepatitis A antibody and appeared to be a likely candidate for the disease. The findings in this Costa Rican family are in accord with the general concept that hepatitis A occurs at very early age in epidemic areas, such as Costa Rica (7, 8). The results of the tests for hepatitis A antibody in the normal young adults in our laboratory (West Point) show a low incidence of hepatitis A antibody (2/22) and a probable lower infection rate than for persons in Costa Rica. Further epidemiologic studies are under way in our laboratories.

The tests for measuring hepatitis A antibody by the CF procedure were complicated by the appearance, at onset of illness, of frequent high level anticomplementary activity that was probably due to presence of antigen–antibody complex. Such reaction

has been observed by others (11) for hepatitis B. They were further complicated by the frequent presence of antibody reacting with normal marmoset liver antigen, though at lower titer and without significant increase except when anticomplementary activity was present. The reaction with normal antigen was probably due, in part at least, to the relatively low viral antigen content of the hepatitis A CF antigen and the need, therefore, to use both antigens at low dilution in the presence of a relatively small dose of complement. The antibody against hepatitis A in human serum was not removed by repeated absorption with normal marmoset liver antigen. It is likely that the CF test for hepatitis A antibody can be improved by use in the test of purified and concentrated preparations of hepatitis A antigen. Even so, the test is highly useful in its present state of development.

It was of some interest that at least one patient (Table I, 068-330-08) who developed antibody against normal liver antigen retained his antibody for at least 122 days. This is suggestive of persistence of antibody that might be worthy of investigation in relation to a possible autoimmune aspect, in some cases, of hepatitis A (12). Such nonspecific antibody might also bear some relationship to the various tests for nonspecific antibody in hepatitis cases reported by several workers (13–21) in years past.

Summary. A specific diagnostic complement-fixation test for hepatitis A antibody in human serum was described employing livers of marmosets infected with CR326 strain human hepatitis A virus. Persons with hepatitis A, but not hepatitis B, developed hepatitis A CF antibody shortly after the onset of illness and this persisted thereafter. Good agreement was noted in the development of CF and neutralizing antibodies in hepatitis A cases. Hepatitis A was shown to occur in a person with hepatitis B antigenemia and hepatitis B occurred in persons with hepatitis A antibody. Most persons with hepatitis A who were tested, but none of those with hepatitis B, developed increased anticomplementary activity in their sera at the time of onset of illness. At least one patient with hepatitis A developed antibody against normal liver that persisted. The possible implications of this in relation to pathogenesis and to nonspecific diagnostic tests in hepatitis were discussed. A limited epidemiologic study of a family outbreak of hepatitis in Costa Rica and of a group of young adults in our laboratory was supportive of the concept that susceptible persons in an epidemic country acquire their infections at an early age and are immune thereafter; persons in areas of relatively low incidence may proceed into adulthood without experience with hepatitis A. The CF test should provide an excellent tool for diagnosis and for epidemiologic investigation of hepatitis A and should be of considerable value to detect hepatitis A virus in attempts to propagate the virus in cell culture.

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