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Specific Immune Adherence Assay for Human Hepatitis A Antibody. Application to Diagnostic and Epidemiologic Investigations (38783)

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Studies of human hepatitis A (infectious hepatitis) have been severely hampered by the lack of a simple assay for specific antibody against hepatitis A virus.

In 1973, we reported (1) the development of a specific serum neutralization test for human hepatitis A antibody using the CR326 virus isolated from a case of human hepatitis. The test, though highly specific, is carried out in marmosets and is too laborious and costly for routine purpose. More recently, we developed (2) a specific complement-fixation (CF) test for hepatitis A antibody employing, as antigen, liver extract of a marmoset infected with the CR326 virus. The test provided a workable *in vitro* assay for serodiagnostic and seroepidemiologic investigations of hepatitis A in man.

The present report describes the development of yet another test for hepatitis A antibody. This is an immune adherence (IA) assay employing, as for the CF tests, extract of liver of marmosets infected with CR326 virus. Data relating to the development of IA antibody against the virus in the course of hepatitis A infection and to the seroepidemiology of the disease are given. Additionally, data relative to the content of hepatitis A antibody in commercial human immune globulin and in various subhuman primate species are presented.

Materials and Methods. Virus. The CR326 strain (3) of human hepatitis A virus isolated in marmosets from a case of hepatitis A in Costa Rica was used. *Preparation of hepatitis A immune adherence (IA) test antigen.* The antigen was prepared from the liver of a marmoset (*Saguinus mystax*) infected intravenously with CR326 virus. The liver was taken 27 days after virus inoculation, at a time when the serum enzymes (SGOT and SICD) were elevated. The liver was perfused with phosphate buffered saline (PBS) at pH 7.2, minced with scissors, and ground with sterile alundum in a mortar to give a final 10% suspension by weight in PBS. The extract was clarified by low speed centrifugation, applied to cesium chloride gradients (4), and fractions in the density range of 1.32–1.36 were collected. The fractions were dialyzed against PBS and were used as antigen in the IA assays. Control antigen was similarly prepared from liver of an uninfected marmoset. *Immune adherence (IA) test.* The procedure was similar to that of Mayumi *et al.* (5) for assay of hepatitis B antigen and antibody. The sera were not heated. The commercial human immune globulins were diluted 1:10 and absorbed with an equal volume of 25% kaolin before they were assayed for antibody content. The antigens were standardized in grid titrations in which serial dilutions of antigen were assayed with serial dilutions of human hepatitis A convalescent serum. Four units of viral antigen were employed in the tests and the control antigen was used at identical dilution. *Complement-fixation (CF) and serum neutralization tests.* The techniques for the CF tests for hepatitis A antibody for hepatitis B surface antigen (HB_sAg or Australia antigen), and for neutralizing antibody were described previously (1, 2).

Patients' sera. Natural hepatitis A and B cases in Costa Rica. These were cases of hepatitis A and 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 carried out there (6, 7). The patients 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 confirmed by standard clinical laboratory tests. All these sera were tested and reported previously for CF antibody (2) and some had been assayed for neutralizing antibody (1, 2) in the marmoset test. *Coded specimens from persons experimentally infected with hepatitis A virus.* The 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 (8, 9). The sera were kindly furnished to us under code by Dr. Krugman. *Normal human sera.* These were collected in routine bleedings from normal persons employed at these laboratories, from commercial blood bank donors in Arizona, or from prisoners in Oklahoma. *Normal animal sera.* These were collected from normal uninoculated animals in our laboratories. *Human immune globulin.* These were standard lots of commercial human immune globulin produced in our laboratories (MSD).

Results. By way of background, persons with hepatitis A but not hepatitis B develop CF (2) and neutralizing (1) antibodies against hepatitis A virus in the course of their infections. Most persons with hepatitis A develop increased anticomplementary CF activity in their sera at time of onset of illness and this is of short duration. Persons with hepatitis A sometimes develop increased CF antibody against normal liver antigen but this is present in far lower titer than antibody against hepatitis A antigen. In the serum neutralization test, a value of 50 or greater was considered significant and the difference in values in comparative tests of acute and convalescent sera was usually 50 or greater (1). The meaning and interpretation of CF and neutralization test results have been described in detail elsewhere (1, 2).

Naturally occurring hepatitis A cases in Costa Rica. The findings in eight cases of hepatitis A that occurred with onset during 1967–68 are presented in Table I. The findings in the first three cases in the table are also presented graphically in Figs. 1, 2 and 3. It is seen that all eight patients developed IA antibody against hepatitis A antigen after onset of acute illness, in one instance within 9 days (204-538-11). These persons also developed CF and neutralizing antibodies against hepatitis A virus. The CF antibody might have appeared earlier than IA antibody but this was confused by the appearance of anticomplementary (AC) activity commonly present early after onset of illness. The IA antibody appeared to be sustained at high level for long periods of time. Sera from two of the cases, 207-056-08 and 068-330-08 were tested for IA antibody using normal liver antigen and all gave negative results. Further, three sequential absorptions of these sera with glutaraldehyde-fixed normal liver antigen did not remove the hepatitis A IA antibody. Finally, heating the sera at 56° for 0.5 hr did not change the test results. *Experimentally infected hepatitis A cases.* The specimens from four cases of hepatitis A shown in Table II were sent to us under code by Dr. Saul Krugman and were tested blindly for CF antibody. All had developed CF antibody. These same sera were tested blindly for IA antibody, but at a time after the code had been broken for the CF test results for cases 1, 2 and 3. Three of the four persons showed a high IA antibody response against hepatitis A. One subject, No. 3, had not so responded by the 70th day after exposure to virus. This might have been due to slower IA than CF antibody response, the last available specimen having been taken only 33 days after onset of enzyme elevation.

Hepatitis B cases. The findings for hepatitis A IA antibody in three cases of hepatitis B, diagnosed by demonstrating HB_sAg (Australia antigen) in the patients' sera, are shown in Table III. None of the persons showed a significant change in IA antibody in the progress of his illness indicating lack of hepatitis A antibody development. The first person in the table appeared to be susceptible to hepatitis A but the remaining two were immunes, having had hepatitis A antibody prior to onset of hepatitis B disease. The IA test results were in agreement with the CF test findings.

Hepatitis A and B. Family epidemiology. Table IV summarizes the occurrence of

TABLE I. IMMUNE ADHERENCE HEPATITIS A ANTIBODY ASSAYS COMPARED WITH PREVIOUS CF AND NEUTRALIZATION TEST RESULTS, HUMAN HEPATITIS A CASES, COSTA RICA.

Case No. (age, yr)	Time of specimen (days)	IA antibody titers	Previous test results (1, 2)			
			CF antibody titers			Hepatitis A neutralization value
			Hepatitis A virus antigen	Normal liver antigen	AC[a]	
207-056-08[b]	−57	<5	20	20	10	
(5 yr, male)	−28	<5	10	20	5	
Fig. 1	0	<5	20	40	5	
	+3	<5	320	320	160	
	+91	40,960	320	40	<5	
	+185	10,240	640	40	5	
068-330-08[b]	−48	<5	5	10	5	−14
(7 yr, male)	−35	<5	10	10	5	
Fig. 2	+2	<5	40	80	40	
	+9	<5	160	80	5	
	+31	5120	640	80	5	
	+122	10,240	1280	40	5	
	+122 +183					89
204-538-11	−20					0
(10 yr, male)	−12	<5	20	40	5	
Fig. 3	−5					0
	+2	<5	80	80	40	51
	+9	1280	320	40	5	
	+90	81,920	640	40	10	
	+90 +178					78
206-033-02	−37					−26
(11 yr, male)	−21	<5	40	80	40	
	+36	20,480	2560	320	160	70
	+102	40,960	2560	40	10	63
203-035-09	−25					20
(8 yr, male)	+3	<5	40	80	20	
	+39					78
	+100	10,240	640	40	5	
702-207-05	−28					10
(4 yr, female)	+14	<5	5	10	5	
	+45	5120	640	20	5	
	+45 +112					91
202-209-07	−28	<5	10	10	5	18
(10 yr, female)	+29 +91					89
	+185	25,600	640	10	<5	
206-343-07[c]	−18	<5	5	10	5	
(8 yr, female)	+9	2560	80	20	<5	
	+183	25,600	80	20	5	

[a] Anticomplementary activity titer.

[b] Sera from the first two cases gave negative IA test results in IA tests with normal marmoset liver antigen. Absorption of the sera three times with glutaraldehyde-treated normal liver antigen did not remove the hepatitis A IA antibody.

[c] This patient was an HB_sAg (hepatitis B) carrier.

hepatitis A and B in a Costa Rican family followed from 3/2/67 through 11/21/74. Case 06, age 9, initially thought to be a hepatitis A index case, showed onset of hepatitis on 3/2/67. It was, however, a likely case of hepatitis B in a person with previous hepatitis A as revealed by the tests for hepatitis B antigen in the sera and

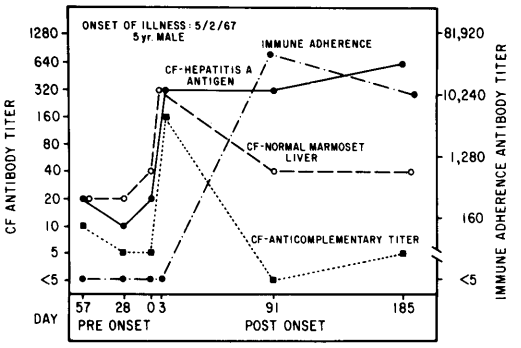

FIG. 1 Hepatitis A CF and IA antibody titers, Costa Rica hepatitis A case # 207-056-08.

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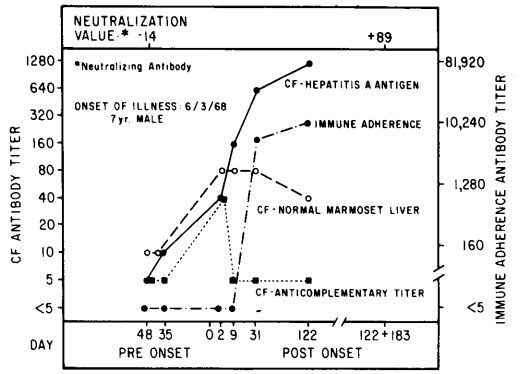

FIG. 2. Hepatitis A CF and IA antibody titers and neutralizing antibody values, Costa Rica hepatitis A case # 068-330-08.

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for hepatitis A antibody. 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 5/2/67. Family members 01, 03, 04, 05 and 07 possessed hepatitis A antibody and were probably immune to reinfection with hepatitis A. Subject 10, age 2, was the youngest member of the family and was without hepatitis A antibody. Surprisingly, subject 02, the mother (age 37), had no antibody against hepatitis A and remained without antibody within the time frame of the study in spite of the occurrence of the disease in the family. Both of these subjects (10 and 02) had developed hepatitis A infection subsequently as revealed by IA and CF antibody in specimens taken November

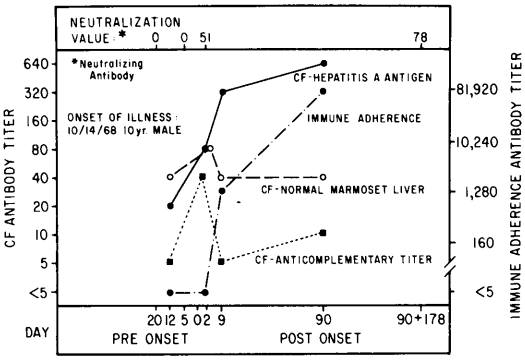

FIG. 3. Hepatitis A CF and IA antibody titers and neutralizing antibody values, Costa Rica hepatitis A case # 204-538-11.

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TABLE II. IMMUNE ADHERENCE HEPATITIS A ANTIBODY ASSAYS COMPARED WITH CF TEST RESULTS, CODED SPECIMENS, HEPATITIS A CASES, IN EXPOSED INDIVIDUALS (BLIND STUDY, CASES OF DR. SAUL KRUGMAN).

Case No.	Day after exposure to virus	SGOT	IA antibody titers	CF antibody titers: Hepatitis A virus antigen	CF antibody titers: Normal liver antigen	CF antibody titers: AC[a]
1	10		<5	80	80	80
	35	Rise[b]				
	59		<5	160	40	20
	71		5,120	640	40	40
	156		10,240	640	80	40
2	13		<5	40	40	40
	30		<5	10	10	10
	37	Rise				
	70		<5	160	40	40
	3¾ yr		10,240	160	<10	<10
3	13		<5	20	20	20
	30		<5	40	80	40
	37	Rise				
	63		<5	320	80	40
	70		<5	160	40	40
4	0		<5	40	40	40
	46	Rise				
	57		<5	160	40	40
	70[c]		40	80	20	10
	1¾ yr		≥10,240	320	40	40

[a] Anticomplementary activity.

[b] First significant elevation in serum SGOT level.

[c] This specimen had dried on storage and was reconstituted to estimated volume.

TABLE III. IMMUNE ADHERENCE HEPATITIS A ANTIBODY ASSAYS COMPARED WITH CF TEST RESULTS, HUMAN HEPATITIS B CASES, COSTA RICA.

Case No. (age, yr)	Time of specimen (days)	IA antibody titers	Previous test results (1, 2)				
			CF antibody titers			Hepatitis A neutralization value	Hepatitis B antigen
			Hepatitis A virus antigen	Normal liver antigen	AC[a]		
202-039-01	−108	<5	20	20	10		0
(36 yr, male)	+10	5	5	10	<5		+
	+149	<5	20	20	10		0
202-039-03	−27	2560	80	10	5		0
(14 yr, male)	+7	5120	80	10	5		+
	+192	2560	40	10	<5		0
202-039-05	−18					50	
(10 yr, female)	−4	640	40	10	5		+
	+8	640	40	20	5		+
	+190	640	40	10	5	67	0

[a] Anticomplementary activity titer.

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

Family 207-056 Member			Date of onset of hepatitis	Date of serum specimens	IA antibody titers	Previous test results (2)			Hepatitis B antigen
						CF antibody titers			
Code	Age (yr)	Sex				Hepatitis A virus antigen	Normal liver antigen	AC[a]	
06[b]	9	M	3/2/67	3/7/67	5120	320	20	5	+
				6/13/67	10,240	320	20	5	+
				11/20/74	2560	160	<5	<5	
09	4	F	4/20/67	3/6/67	<5	20	40	10	0
				11/6/67	10,240	640	20	5	0
				11/20/74	5120	320	<5	<5	
08	5	M	5/2/67	3/6/67	<5	20	40	<5	0
				8/4/67	40,960	320	40	<5	0
				11/20/74	5120	320	<5	<5	
01	40	M	Not ill	3/6/67	1280	80	40	<5	0
				5/30/67	5120	40	40	<5	0
				11/21/74	2560	160	<5	<5	
02	37	F	Not ill	3/6/67	<5	40	40	20	0
				5/30/67	<5	40	40	20	0
				11/20/74	1280	160	5	5	
03	14	F	Not ill	3/6/67	2560	160	40	10	0
				6/1/67	2560	40	40	10	0
				11/21/74	10,240	160	<5	<5	
04	13	M	Not ill	3/13/67	2560	80	20	<10	0
				5/30/67	1280	80	80	10	0
				11/20/74	1280	160	<5	<5	
05	10	F	Not ill	3/6/67	5120	160	10	5	0
				5/30/67	5120	160	20	10	0
				11/20/74	10,240	640	5	5	
07	7	M	Not ill	3/6/67	5120	160	20	5	0
				5/30/67	2560	160	80	10	0
				11/21/74	2560	160	<5	<5	
10	2	M	Not ill	3/6/67	<5	10	20	<5	0
				5/30/67	<5	10	20	<5	0
				11/21/74	320	80	5	<5	

[a] Anticomplementary activity titer.

[b] Case 06 was icteric with enzyme elevation; Case 09 was clinically anicteric hepatitis with enzyme elevations; Case 08 was subclinical hepatitis but with enzyme elevations.

TABLE V. HEPATITIS A IMMUNE ADHERENCE TITERS IN NORMAL PERSONS.

IA Titer	No. persons with titer: Young adults West Point, PA	Blood bank donors	Prisoners
<10	19	22	10
10	0	0	0
20	0	0	0
40	0	1	1
80	0	1	0
160	0	1	4
320	0	1	0
640	1	1	8
1280	1	2	0
2560 or >	1	4	3
No. pos./ total	3/22	11/33	16/26
% pos.	14%	33%	62%

20 or 21, 1974. Importantly, both IA and CF antibody were retained for at least 7 yr without substantial change. *Normal human sera.* The findings for hepatitis A IA antibody in bleedings taken routinely from normal persons are shown in Table V. It is seen that only 3 of 22 (14%) young adult persons in these laboratories had antibody. One of the subjects with antibody was from Puerto Rico, one had worked as a technician in a blood bank prior to present employment, and one had a history of previous serum enzyme elevation but without clinical hepatitis. The blood bank donors in Arizona, who were generally persons of low socioeconomic status, showed 33% to have hepatitis A antibody and 62% of the inmates of a prison in Oklahoma also had such antibody. Where present, the IA antibody titer was often high and most exceeded 1:320. *Human immune globulins.* As might be expected, all of 24 lots of human immune globulin (results shown in Table VI) had hepatitis A IA antibody. There was 16-fold difference in antibody titer between the various lots. The highest titer in the lots was 1:16,000 and most lots had titers of 1:4000 or 1:8000.

Normal animal sera. The findings for hepatitis A IA antibody in the sera of normal subhuman primates, rodents, and swine are shown in Table VII. The rodents, swine, and four of the subhuman primate species were without antibody. Importantly, a portion of the chimpanzees, grivet (*Cercopithecus aethiops*) and rhesus (*Macacus rhesus*) monkeys had hepatitis A antibody.

Discussion. The reliable propagation of CR326 strain human hepatitis A virus in marmosets (1, 3) permitted the development of methods for *in vitro* assay of hepatitis A antigen and antibody. The present report describes the development and application of a simple immune adherence assay for hepatitis A antibody employing, as antigen, purified CR326 strain human hepatitis A virus from infected marmoset liver. The antigen, when combined with antibody and complement, aggregates human 0 erythrocytes. Such reaction is not found when normal marmoset liver antigen is used nor is the hepatitis A antibody removed on

TABLE VI. DISTRIBUTION, HEPATITIS A IMMUNE ADHERENCE TITERS, 24 COMMERCIAL LOTS OF MSD HUMAN IMMUNE GLOBULIN.

IA Titer	No. lots
1000	1
2000	2
4000	11
8000	9
16,000	1

TABLE VII. RESULTS OF IA TESTS FOR HUMAN HEPATITIS A ANTIBODY IN NORMAL ANIMALS.

Animal	No. positive / Total tested
Chimpanzee[a]	14/23 (61%)
Grivet[a]	9/40 (23%)
Rhesus[a]	1/17 (6%)
Baboon	0/6
Gibbon	0/1
Spider monkey	0/1
Marmoset (*S. mystax*)	0/82
Mini-pigs (swine)	0/6
Rat	0/6
Hamster	0/6
Guinea pig	0/45

[a] Titers in positive chimpanzees were 1:40 to >1:6400; they were 1:40 to 1:5120 in grivets and 1:2560 in the one rhesus monkey.

repeated absorption with normal marmoset liver antigen. The development of the CF (2) and the serum neutralization (1) tests were described earlier.

In the study reported here, all of seven suspected cases of human hepatitis A and one case of hepatitis A, initially misdiagnosed as hepatitis B because of hepatitis B (HB_sAg) antigenemia, developed IA antibody in the course of their disease. The findings in the IA assay were confirmed by the CF and neutralizing antibody findings in tests performed on the same sera. The time of first appearance of IA antibody was not precisely defined but, like neutralizing and CF antibodies, was present soon after onset of illness. In two instances, (Table I, cases 207-056-08 and 068-330-08), CF antibody may have been present before IA antibody but this was obscured by anticomplementary activity. In one case (Table I, 206-343-07), both CF and IA antibodies were present 9 days after onset of acute hepatitis. The data indicate that maximal IA antibody titers were reached within 2 or 3 mo after onset of clinical hepatitis, perhaps even within one month (Table I, cases 068-330-08 and 206-033-02). IA antibody titers persisted for as long as 7 yr, the longest period tested (Table IV).

The validity of the hepatitis A IA antibody test results was supported by several evidences. There was remarkable agreement in the results of tests obtained for IA, CF and neutralizing antibodies. Persons with hepatitis B did not develop hepatitis A IA antibody and they did not develop CF or neutralizing antibody against hepatitis A. Further support was given in the findings with coded sera sent to us for blind testing by Dr. Saul Krugman. In these tests, all four persons infected with hepatitis A virus developed CF antibodies against the agent. Three of the four developed IA antibody. The failure of the fourth person to develop such antibody may have been due to the fact that the latest available blood sample was taken only 33 days after first elevation in SGOT and this might have been too early to detect IA antibody.

The IA test appears to be far more sensitive in detecting hepatitis A antibody than is either the CF or neutralization test. IA titers rise to levels of 1:40,960, while the highest CF titer found to date was 1:2560. The neutralization test, as performed with marmosets, is, by its nature, a less quantitative and relatively insensitive assay for neutralizing antibody content.

In the seroepidemiologic study in the one Costa Rican family, it was possible to detect active hepatitis A or B disease in three persons in the family. It appeared that hepatitis A was largely an event of early life. The disease had not yet occurred in the infant, age 2, and all but one of the remaining persons 7 yr or older were immune. Surprisingly, the mother, aged 37 yr, had no antibody. Both mother and infant subsequently developed hepatitis A, as judged by the presence of CF and IA antibody in sera taken 7 yr later. Both IA and CF antibody were retained for at least 7 yr after illness without substantial change in titer, suggesting that immunity to hepatitis A is long-lasting. Though generalization based on a single family is not possible, the findings are in accord with the general concept that hepatitis A tends to be a disease of persons of young age in countries in which the disease is epidemic—such as in Costa Rica (6, 7). The tests for antibody in normal young adults in our laboratory, in an area in which hepatitis A is less frequent, suggest high susceptibility to hepatitis A, even into adulthood. Importantly, persons of lower socioeconomic background, such as commercial blood bank donors and prisoners, had far more frequent experience with hepatitis A virus.

The application of the hepatitis IA test makes possible the control for potency for hepatitis A antibody of human immune globulin. There was considerable variation of potency of individual commercial lots of globulin though most titered 1:4000 or 1:8000.

The limited tests of rodent and swine sera gave no indication of infection with human hepatitis A virus. Among the small numbers of each of seven subhuman primate species tested, chimpanzees and grivet and rhesus monkeys alone had antibody. The chimpanzee findings are not surprising in view

of the suspected susceptibility of these animals to human hepatitis A (10). The grivet and rhesus monkey findings are less expected and they suggest the susceptibility of these species to human hepatitis A virus. There is the possibility, however, that these subhuman primates might be infected naturally with indigenous viruses that are related antigenically to human hepatitis A virus. These primate species are currently being tested in our laboratories for susceptibility to CR326 human hepatitis A virus. Our negative results reported for marmosets were with animals not given hepatitis A virus. Marmosets recovered from hepatitis A infection do have hepatitis A antibody (11).

Summary. A specific immune adherence (IA) test for hepatitis A antibody in human serum was described employing liver extract of marmosets infected with CR326 strain human hepatitis A virus. Persons with hepatitis A, but not hepatitis B, developed hepatitis A IA antibody soon after onset of the acute illness and this persisted thereafter. There was very close agreement in the tests for human hepatitis A immune adherence, complement fixing (CF) and neutralizing antibodies. IA antibodies appeared to develop somewhat later than CF or neutralizing antibody. A limited epidemiologic study of a family outbreak of hepatitis A and B in Costa Rica showed simultaneous occurrence of the two diseases and was supportive of the concept that susceptible persons in a country with high hepatitis A prevalence generally acquire their infections at an early age and are immune thereafter. Most persons of high socioeconomic level in an area of low hepatitis A incidence may proceed to adulthood without experience with hepatitis A. Persons of low socioeconomic level, however, such as commercial blood bank donors and prisoners, show high incidence of hepatitis A antibody. Hepatitis IA and CF antibodies persisted in human subjects for at least 7 hr after hepatitis A virus infection. Captive chimpanzees and grivet and rhesus monkeys, not given hepatitis A virus, showed evidence of previous experience with human hepatitis A or an antigenically related virus based on tests for hepatitis A antibody. Other subhuman primates, rodents, and swine, not given hepatitis A virus, were without hepatitis A antibody. The IA test provides an excellent tool for diagnostic and epidemiologic investigations of hepatitis A and should be of considerable value to detect hepatitis A virus in attempts to propagate the virus in cell culture. There was considerable difference in hepatitis A IA antibody content of different lots of commercial human immune globulin, though the majority titered 1:4000 or 1:8000.

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1. Provost, P. J., Ittensohn, O. L., Villarejos, V. M., Arguedas G., J. A., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **142**, 1257 (1973).
2. Provost, P. J., Ittensohn, O. L., Villarejos, V. M., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med., **148**, 961 (1975).
3. Mascoli, C. C., Ittensohn, O. L., Villarejos, V. M., Arguedas G., J. A., Provost, P. J., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **142**, 276 (1973)
4. Provost, P. J., Wolanski, B. S., Miller, W. J., Ittensohn, O. L., McAleer, W. J., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med., **148**, 532 (1975).
5. Mayumi, M., Okochi, K., and Nishioka, K., Vox. Sang. **20**, 178 (1971).
6. Villarejos, V. M., Arguedas, J. A., Gutierrez, A., Eduarte, E., Vargas A., G., and Osborne, J. A., Amer. J. Epidemiol. **96**, 361 (1972).
7. Villarejos, V. M., Gutierrez, A., and Pelon, W., Amer. J. Epidemiol. **96**, 372 (1972).
8. Krugman, S., Giles, J. P., and Hammond, J., J. Amer. Med. Ass. **200**, 365 (1967).
9. Krugman, S., and Giles, J. P., J. Amer. Med. Ass. **212**, 1019 (1970).
10. Center for Disease Control Hepatitis Surveillance, Report. No. 34, U.S. Department of Health, Education, and Welfare, Public Health Service, Health Services and Mental Health Administration, September (1971).
11. Provost, P. J., Ittensohn, O. L., and Hilleman, M. R., unpublished results.

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