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Serodiagnosis of Viral Hepatitis A: Detection of Acute-Phase Immunoglobulin M Anti-Hepatitis A Virus by Radioimmunoassay

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A modified micro solid-phase radioimmunoassay (RIA) for antibody to hepatitis A virus (anti-HAV) was developed. This double antibody procedure was performed by coating the surface of a polyvinyl microtiter plate "well" with 200 μl of a 1:1,000 dilution of a patient's test serum. Purified HAV and ^{125}I-labeled immunoglobulin G (IgG) anti-HAV were then sequentially added to form an antibody sandwich. The specificity and sensitivity of the RIA procedure for anti-HAV were verified by examination of coded human and chimpanzee serum specimens. Radioimmunoassay of early-acute-phase serum specimens from human cases of hepatitis A revealed the presence of anti-HAV activity. Differential examination by RIA of IgG and IgM fractions of acute-phase sera from experimentally infected chimpanzees demonstrated that IgM contained the bulk of the anti-HAV activity. A modification of the RIA procedure for anti-HAV (RIA-IgM blocking), incorporating an incubation step with anti-IgM (Mu chain specific), was further shown to differentiate acute- from convalescent-phase hepatitis A sera. This adapted RIA-IgM blocking procedure required less than 1 μl of a single acute-phase serum specimen for the diagnosis of viral hepatitis A.

The visualization by immune electron microscopy (IEM) of 27-nm virus-like particles in acute-phase stools of volunteers infected with the MS-1 strain of hepatitis A virus (HAV) (5) provided the impetus for subsequent studies of hepatitis A in human populations and in subhuman primates. The discovery by IEM of morphologically and immunologically similar 27-nm virus-like particles in a stool pool obtained from acutely ill individuals involved in a common source outbreak of hepatitis A (6) provided confirmatory evidence that these particles were HAV. Marmoset monkeys inoculated with the MS-1 strain of HAV developed enzymatic and histopathological evidence of viral hepatitis (12). Their acquisition of specific humoral antibody (anti-HAV) was documented by IEM and provided further evidence for their susceptibility to infection with HAV. Chimpanzees inoculated with chimpanzee and human stool extracts containing HAV were also shown to be susceptible to infection, as judged by characteristic rises in serum glutamic pyruvic transaminase (SGPT) activity 17 to 38 days after inoculation and by coincident liver histopathology indicative of viral hepatitis (10). HAV was detected by IEM in liver, bile, and stools during the preacute- and early-acute phases of infection (1, 17). Development of serum anti-HAV was also documented by IEM (3, 10) and by a newly developed solid-phase radioimmunoassay (RIA) (8).

Significant advances in our understanding of viral hepatitis A have been achieved through the development and application of new serological techniques for the detection of both HAV and anti-HAV. Many of these studies have been performed with reagents derived from humans as well as from marmosets and chimpanzees. The serological techniques include complement fixation (15), immune adherence hemagglutination (IAHA) (13, 14), and micro solid-phase RIA procedures (7, 8, 16). For detection of anti-HAV, these procedures required HAV purified (or partially purified) from human or chimpanzee feces, or from infected marmoset livers. We now describe a micro solid-phase, double-antibody RIA procedure for detection of anti-HAV that employs limited quantities of HAV purified from chimpanzee feces. The sensitivity and specificity of this RIA test and an adaptation of the RIA procedure for the serodiagnosis of acute viral hepatitis A are discussed below.

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MATERIALS AND METHODS

RIA for HAV. HAV was detected in chimpanzee stool preparations by RIA (8). Briefly, 200 μl (1 to 2 μg) of chromatographically purified immunoglobulin G (IgG; human) containing anti-HAV activity was used to coat the wells of a polyvinyl microtiter "U" plate (no. 220-24, Cooke Engineering Co., Alexandria, Va.). The plate was covered with Parafilm and was incubated for 3 h at room temperature, then overnight at 4°C in a humidified environment. At the end of the overnight incubation period, the wells were washed two times with 0.01 M phosphate-buffered saline (PBS) (pH 7.2) containing 2% fetal calf serum (PBS-FCS). A 50-μl portion of the sample to be tested for HAV was added to each of two wells, and the plate was incubated by flotation in a water bath at 45°C for 2 h. The contents of the wells were then aspirated by suction, and the wells were washed five times with PBS-FCS. After washing, 50 μl (100,000 cpm) of ^{125}I-labeled and chromatographically purified IgG (specific activity, 1 to 2 atoms of ^{125}I per molecule of IgG) containing anti-HAV activity was added to each well, and the plate was incubated for 90 min at 45°C. After an additional washing step to remove unbound labeled antibody, individual wells were cut out with scissors and counted in a gamma counter for 10 min or for a total of 4,000 counts. The residual mean counts per minute of at least eight control samples devoid of HAV was used to determine the mean background counts per minute, N. Any test sample counts per minute (P) in excess of $2.1 \times N$ was considered positive for HAV.

Purification of HAV. HAV particles were purified from preacute-phase chimpanzee feces by a combination of polyethylene glycol precipitation, cesium chloride banding, and molecular sieve chromatography (2, 9). These three procedures take advantage of the fact that HAV is a nucleoprotein, possesses a buoyant density that is significantly different from that of the bulk of the stool material, and has a particle diameter that excludes it from more than 99% of the material in stools when chromatographed on Sepharose 2B. The stools used for the purification of HAV were obtained from an experimentally infected chimpanzee on days 14 and 15 after inoculation with HAV (10). An initial 17% weight/volume (wt/vol) crude stool suspension was prepared by combining 20 g of stool with 100 ml of water. The suspension was clarified at $3,000 \times g$ in a fixed-angle rotor. The supernatant fluids were carefully removed from the crude pellets and combined with 7% or 10% wt/vol polyethylene glycol to precipitate HAV. The resulting floculant mass was centrifuged at $18,000 \times g$ for 25 min at 5°C. The pellets were suspended in 20 ml of 0.05 M PBS, pH 7.2. A 4-ml portion of the fivefold polyethylene glycol-concentrated preparation was layered onto a 34-ml linear cesium chloride density gradient with a buoyant density range of 1.174 to 1.500 g/cm^3. The gradient was centrifuged in an SW27 swinging bucket rotor for 23 h at 5°C. Thirty-two 1.2-ml fractions were collected and assayed for the presence of HAV by the RIA procedure described above. Cesium chloride gradient fractions containing the bulk of the HAV activity (buoyant density, 1.33 to 1.34 g/cm^3) were pooled and subjected to Sepharose 2B gel filtration. The sample (2 ml) was chromatographed through a glass column (1.6 by 70 cm) by gravity flow with 0.1 M tris(hydroxymethyl)aminomethane-2.0 M sodium chloride buffer, pH 7.2. Seventy 2.0-ml fractions were collected and assayed for HAV by RIA. Immune electron microscopic and direct electron microscopic examination of the peak Sepharose 2B fractions revealed the presence of highly purified HAV particles (2).

RIA for anti-HAV. Sera to be screened for anti-HAV were first diluted 1:1,000 in a separate microtiter plate. Three wells were filled with 225 μl of 0.01 M PBS (pH 7.2) containing a 1:4,000 dilution of sodium azide as preservative. A 25-μl portion of the serum to be tested for anti-HAV was added to the first well and looped out to the third well with a 25-μl microdiluter. When sera were titrated through more dilutions, pipettes, rather than loops, were used. A 200-μl amount of the diluted test serum was then transferred to a test plate. The test plate was covered and incubated at room temperature (approximately 25°C) overnight. After incubation, the plate was washed two times with PBS-FCS. A 50-μl sample of purified HAV was added to each test sample. The plate was again covered and incubated at room temperature overnight (18 h). After the second incubation, the plate was washed five times with PBS-FCS. A 50-μl portion of ^{125}I-labeled IgG anti-HAV (100,000 cpm) was added to each test well. The plate was again covered and incubated by flotation in a 37°C water bath for 4.0 h. After the third and final incubation step, the plate was removed from the water bath and washed five times with PBS-FCS. The plate was dried, and the wells were cut out with scissors and dropped into gamma-counting tubes and counted for 10 min or a total of 4,000 counts. At least seven sera known to be negative for anti-HAV were assayed along with the test sera and were used as the negative controls. The mean counts per minute from these anti-HAV negative sera was designated N. P was the counts per minute of the test sample. Any test sample with a P/N ratio equal to or greater than 2.1 was considered positive for anti-HAV. P/N ratios for test sera normally ranged between 0.7 and 30.0, depending upon the antibody content of the serum specimen and the quantity of HAV added to the test well. To assure at least a moderate degree of comparability between one test for anti-HAV and any subsequent test, reagent HAV was titrated for each set of tests. Fifteen N units of HAV were normally used for each routine antibody assay. That is, if N equaled 100 cpm, then a dilution of hepatitis A antigen, which yielded 1,500 cpm in a standard antigen test, was used in the antibody assay. P/N ratios greater than 15 were possible in the anti-HAV test since some test sera had anti-HAV potency greater than that found in the standard serum (or IgG) used to coat the microtiter well.

RIA for IgM-specific anti-HAV (RIA-MB). Acute-phase sera were assayed for IgM anti-HAV by a modification of the RIA procedure. Acute-phase sera from patients and experimentally infected

chimpanzees were diluted to 1:5,000, 1:10,000, 1:50,000, and 1:100,000 in 0.01 PBS, pH 7.2. Four 100-μl samples of each serum dilution were added to separate wells of a polyvinyl microtiter plate and incubated overnight at room temperature. After washing with PBS-FCS, two wells of each serum dilution separately received 100 μl of normal goat serum (NGS) diluted 1:500 and 1:1,000 in PBS, whereas the remaining two wells separately received 100 μl of goat antiserum to human IgM (GAS) (Mu chain specific, 7.1 mg/ml; Behring Diagnostics, Somerset, N.J.) diluted 1:500 and 1:1,000 in PBS. The microtiter plate was covered and again incubated overnight at room temperature. After washing, purified HAV and ^{125}I-labeled IgG anti-HAV were sequentially added to the wells, as described previously. The individual wells were cut out and counted as described above. Seven control wells received 0.01 M PBS (pH 7.2)-1.0% bovine serum albumin in place of the patient's diluted test serum. Residual counts per minute from these wells were used to compute mean background counts per minute. Mean background counts per minute were subtracted from each of the gross counts per minute to yield specific counts per minute. A 30% or greater reduction of test sample-specific counts per minute by goat anti-IgM (compared with normal goat serum) was considered diagnostic for the presence of IgM anti-HAV.

Sources of serum. Serum collections used in this investigation were derived from several sources. The first collection (panel 1) consisted of preinoculation, early-acute phase, late-acute phase, and convalescent-phase sera obtained from nine chimpanzees experimentally infected with HAV. All nine animals exhibited abnormal SGPT elevations between 17 and 40 days after inoculation with HAV and demonstrated histopathological evidence consistent with a diagnosis of viral hepatitis. Panel 2 was provided by Jules Dienstag, National Institutes of Health, and consisted primarily of acute- and convalescent-phase sera from patients with hepatitis A or B. Panel 3 was provided by Arie Zuckerman, London School of Hygiene and Tropical Medicine, and consisted of serial serum specimens obtained from a chimpanzee experimentally infected with HAV, as well as specimens from human patients with recurrent familial cholestatic jaundice, or who were in the convalescent phase of hepatitis A. Additional sera were from two chimpanzees, three sheep, and one goat. Panel 4 consisted of sera obtained from 24 icteric cases of hepatitis A during a common source outbreak of this disease at an institution for the mentally retarded in New England. The epidemiological investigation of this outbreak is reported in detail elsewhere (S. H. Hindman, J. E. Maynard, D. W. Bradley, A. E. Denes, and K. R. Berquist, Am. J. Epidemiol., in press). These sera represented specimens collected prior to the onset of jaundice, during jaundice, and in convalescence.

IgG and IgM preparations. Separation of IgG and IgM from a plasmapheresis sample obtained from a chimpanzee in the acute phase of hepatitis A infection was performed by molecular sieve column chromatography. A 15-ml portion of plasma taken from an HAV-infected chimpanzee on day 19 postinoculation (SGPT peak on day 21) was defibrinated and applied to a column (2.6 by 100 cm) packed with Sepharose 6B (Pharmacia). The sample was eluted with 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride-0.2 M NaCl, pH 8.0; 3-ml fractions were collected by gravity flow at a rate of about 2 ml/cm^2 $\times$ h. The fractions containing higher molecular-weight species, i.e., IgM, and showing no traces of IgA or IgG by radial immunodiffusion in commercially available plates (Behring), were pooled and reconcentrated to the original serum IgM concentration by ultrafiltration. Column fractions containing IgG (free of IgM) were also pooled and concentrated by ultrafiltration to the original serum concentration. Examination of the concentrated IgG and IgM preparations by radial immunodiffusion and immunoelectrophoresis revealed no contamination with other immunoglobulins, i.e., no IgA or IgM in the IgG preparation and no IgA or IgG in the IgM preparation.

RESULTS

RIA for anti-HAV: effect of serum dilution on reactivity. Preinoculation-, early-acute-phase, late-acute-phase, and convalescent-phase sera (Table 1) obtained from seven HAV-infected chimpanzees included in panel 1 were tested at serial 10-fold dilutions for the presence of anti-HAV. The results for sera obtained from

TABLE 1. *Development of anti-HAV in experimentally infected chimpanzees*

Animal no.	RIA *P/N* ratio[a]			
	Preinoculation	Acute-1[b]	Acute-2[c]	Convalescent[d]
0754	1.1	6.7	NT[e]	17.0 (54)
0722	0.7	6.2	6.0	22.9 (56)
661	1.0	6.5	11.4	24.2 (70)
0084	0.7	12.5	12.9	32.6 (515)
0082	1.4	0.7	7.8	18.6 (61)
752	0.9	15.2	16.2	23.6 (350)
770	1.5	12.8	12.9	19.6 (70)
0090	0.8	1.0	9.3	NT[f]
0077	0.9	0.9	12.6	NT[f]
Avg *P/N*	1.0	6.9	11.1	22.6

[a] N = 147 cpm (mean counts per minute of nine preinoculation sera); all P/N ratios computed for test serum dilution of 1:1,000. 15 N units of HAV were used per assay.

[b] Preacute- or early-acute-phase sera were obtained just prior to (within 3 days) or at the time abnormal levels of SGPT activity were detected.

[c] Acute-phase sera were obtained 10 to 14 days after elevated SGPT activity was detected.

[d] Convalescent-phase serum was obtained on day indicated (days after inoculation shown in parentheses).

[e] Nt, Not tested.

[f] Animal expired in acute phase of disease.

one chimpanzee (Fig. 1) clearly demonstrated the effects of serum dilution on the reactivity of anti-HAV in the double-antibody sandwich RIA and were typical of the findings for all seven chimpanzees. The preinoculation serum yielded low and nearly constant counts per minute at dilutions of 1:10 through 1:10,000,000. Early-acute- and late-acute-phase sera were positive for anti-HAV at dilutions of 1:10 through 1:100,000. The convalescent serum, however, was negative for anti-HAV at a dilution of 1:10, but was positive for antibody at dilutions of 1:100 through 1:10,000,000. Maximum reactivity of the chimpanzee acute- and convalescent-phase sera occurred at dilutions of 1:1,000 and 1:10,000. These results clearly demonstrate the existence of a marked prozone-like effect at low serum dilutions and provide the basis for our decision to screen sera at an optimal starting dilution of 1:1,000.

Development of anti-HAV in infected chimpanzees (panel 1). All chimpanzee sera from panel 1 were screened in triplicate at a uniform dilution of 1:1,000. Fifteen *N* units of purified HAV were used in the assays. All nine preinoculation sera (Table 1) yielded low counts per minute, with a mean of 147 cpm (standard deviation ± 42 cpm). By using a negative control cut-off of 309 cpm (2.1 × 147), six of the nine early-acute-phase sera (acute-1) were judged positive for anti-HAV, whereas all of the late-acute-phase (acute-2) sera tested were found to be positive for anti-HAV. In seven chimpanzees for which convalescent sera were available, RIA also detected the presence of anti-HAV activity. IEM examination of the nine preinoculation- and seven convalescent-phase sera provided confirmatory evidence that all preinoculation sera were negative for antibody to the HAV particles, whereas all convalescent-phase sera contained antibody specific for HAV (4+ on a scale of 0 to 4). IEM examination of early-acute- and late-acute-phase sera for anti-HAV also confirmed the RIA results, since lower levels of antibody (1 to 3+) were found associated with RIA-positive samples demonstrating *P/N* ratios lower than those observed with matching convalescent-phase sera (unpublished data). Most notable was the development of anti-HAV in the early stages of disease; two-thirds of the infected chimpanzees were anti-HAV positive by the time elevated SGPT activity was first detected.

It should also be noted (Table 1) that RIA *P/N* ratios were generally found to increase between the early- and late-acute phases, as well as between the late-acute and convalescent phases of disease. Figure 2 illustrates the temporal course of anti-HAV development in three of the nine experimentally infected chimpanzees. Sera were found to be positive for anti-HAV just before, or at the time of initially elevated, SGPT activity. Anti-HAV *P/N* ratios were lower in the early stages of disease, days 12 through 21, but tended to increase with increasing time beyond the acute-illness phase. Chimpanzee 0084 day 515 serum, for example, yielded an RIA *P/N* ratio of 33. Late-convalescent sera were not available from the other two chimpanzees. IgG and IgM fractions from

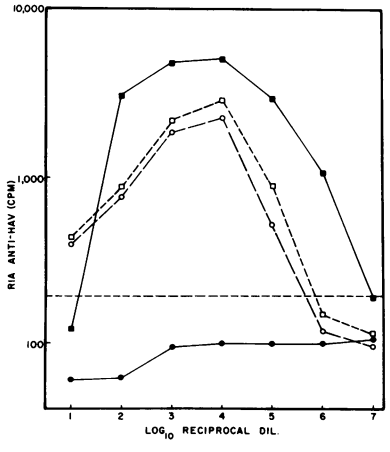

FIG. 1. *RIA for anti-HAV: effect of serum dilution on reactivity. Thirty-five N units of HAV (3,255 cpm) were used for each assay. N = 93 cpm at a preinoculation serum dilution of 1:1,000. Sera: ●, preinoculation; ○, early-acute phase; □, acute phase; ■, convalescent phase. Dashed horizontal line represents 2.1 × N = 195 cpm. Sera were obtained from chimpanzee 0084.*

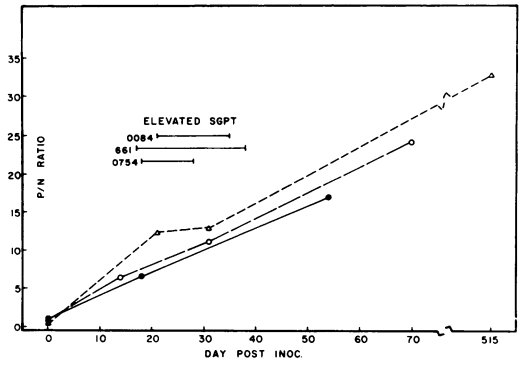

FIG. 2. *RIA for anti-HAV in preinoculation-, early-acute-, late-acute-, and convalescent-phase chimpanzee sera. Sera from chimpanzees 0084, 661, and 0754 were diluted 1:1,000 and tested for anti-HAV. Fifteen N units of HAV were used in each assay. Sera: △, animal 0084; ○, animal 661; ●, animal 0754.*

early-acute-phase sera from two chimpanzees were examined by RIA for anti-HAV. Examination of the two immunoglobulin fractions from both animals yielded similar RIA results. Data from one of the animals are illustrated in Figure 3. The negative control for the RIA test was 0.01 M PBS (pH 7.2) containing 1% bovine serum albumin. IgG was maximally reactive in the RIA test at a dilution of 1:100 and demonstrated an anti-HAV titer of 1:1,000 to 1:10,000. IgM exhibited a maximum *P/N* ratio at a dilution of 1:10,000 and had an anti-HAV titer greater than 1:1,000,000. These data confirm the specificity of the early anti-HAV IgM response in the infected chimpanzees and show that the IgM fractions exhibited a much higher anti-HAV activity than did the IgG fractions.

Serum panels 2 and 3. Additional evidence for the specificity of the RIA test for anti-HAV was provided by examination of panels of coded sera. Panel 2 (Table 2) contained precoded, acute-illness- and convalescent-phase sera from human cases of hepatitis A or B, as well as one convalescent (hepatitis A) chimpanzee serum and one human serum with anti-HBc. Three pairs of acute-illness- and convalescent-phase sera were from patients with hepatitis B, and two pairs were from patients with hepatitis A. One pair of hepatitis A sera (16 and 14) were duplicate samples of sera 5 and 6. All sera had been previously tested at the National Institutes of Health for anti-HAV activity by IAHA. All IAHA-positive convalescent hepatitis A sera were found to be positive for anti-HAV by RIA. All seven of the acute hepatitis A sera were also judged positive for anti-HAV by RIA. Here, the IAHA results were in partial agreement with the RIA results for acute-phase anti-HAV, since only four of the seven sera were shown to contain anti-HAV by the hemagglutination method. Paired sera from patients with hepatitis B, 19 and 2 and 9 and 17, were shown by IAHA and by RIA to contain preexisting anti-HAV. The remaining pair of hepatitis B sera, 15 and 12, was negative for anti-HAV by

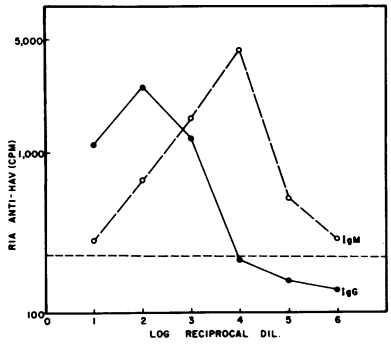

FIG. 3. *RIA for anti-HAV in IgG (●) and IgM (○) fractions of an acute-phase hepatitis A plasma. Plasma from chimpanzee 722 (day 19 postinoculation; SGPT peak, day 26) was defibrinated and subjected to molecular sieve column chromatography, as described in the text. IgG and IgM fractions (reconcentrated by ultrafiltration to their original plasma concentrations of 14.3 and 2.1 mg/ml, respectively) were tested for anti-HAV at serial 10-fold dilutions. Fifteen N units of HAV were used for each assay (N = 109 cpm). Dashed horizontal line represents 2.1 × N (229 cpm), used as the negative cut-off boundary.*

TABLE 2. *Coded serum panel 2: IAHA and RIA for anti-HAV*

Code no.	Description	IAHA titer[a]	RIA *P/N* ratio[b]
1	Acute hepatitis A	100	50.9
[19[c]	Acute hepatitis B	1,280	20.3
[2	Convalescent hepatitis B	640	22.2
[9	Acute hepatitis B	1,280	22.1
[17	Convalescent hepatitis B	640	20.6
[15	Acute hepatitis B	<10 (neg)	1.3
[12	Convalescent hepatitis B	<10 (neg)	1.2
7	Human anti-HBc	<10 (neg)	1.0
[5	Acute hepatitis A	<10 (neg)	62.9
[8	Convalescent hepatitis A	320,000	41.1
[20	Acute hepatitis A	4,000	57.4
[18	Convalescent hepatitis A	80,000	38.6
3	Acute hepatitis A	<10 (neg)	76.7
21	Convalescent hepatitis A	16,000	63.2
[16 (5)[d]	Acute hepatitis A	<10 (neg)	66.7
[14 (8)	Convalescent hepatitis A	320,000	43.8
13 (1)	Acute hepatitis A	100	47.6
11	Phoenix acute hepatitis A[e]	20,000	74.6
10	Convalescent hepatitis A	320,000	44.8
6	Convalescent hepatitis A	2,000	72.6
4	Chimp convalescent hepatitis A	3,200	30.3

[a] IAHA results kindly provided under code by Jules Dienstag. IAHA was performed using HAV partially purified from human acute-phase stools.

[b] *N* = 127 cpm (mean of seven negative sera). All *P/N* ratios were computed for serum dilution of 1:1,620; 30 *N* units of HAV were used per assay.

[c] Brackets indicate paired acute-illness- and convalescent-phase sera.

[d] Coded sera 16 and 14 were duplicate samples of sera 5 and 8, respectively.

[e] Acute-illness-phase serum from Phoenix (common source outbreak of hepatitis A).

IAHA and by RIA. In addition, human serum containing anti-HBc was also shown to be negative for anti-HAV by both serological tests. RIA *P/N* ratios for the coded sera pair 5 and 8 (62.9 and 41.1, respectively) were in excellent agreement with the *P/N* ratios of the duplicate samples 16 and 14 (66.7 and 43.8, respectively).

Serum panel 3 (Table 3) contained precoded experimental goat and sheep sera, chimpanzee sera (including 11 serial specimens from an HAV-infected animal), human convalescent hepatitis A sera, and two sera from patients with recurrent familial cholestatic jaundice. All but three sera had been previously tested by IEM in London for anti-HAV. All sera originally found positive by IEM for anti-HAV were also judged positive by RIA. With only one possible exception (serum 36827), all sera found negative by IEM for anti-HAV were also found negative by RIA. Closer examination of Table 3 reveals that chimpanzee George developed anti-HAV by at least 30 days after inoculation. All sera obtained between days 30 and 272 after inoculation were found positive for anti-HAV by both IEM and RIA. Sera from two patients with recurrent familial cholestatic jaundice were shown to be negative for anti-HAV by IEM and RIA. Sera from four ungulata (3 sheep, 1 goat) experimentally immunized with HAV-positive materials were uniformly negative for anti-HAV by IEM. RIA, however, detected possible low levels of anti-HAV in one of the sheep sera, 36827.

Common-source outbreak of hepatitis A. The results of anti-HAV testing of paired sera from 24 cases of hepatitis A associated with an outbreak in a home for the mentally retarded (panel 4) are summarized in Table 4. Anti-HAV was detected by the RIA technique in all sera from these cases collected on or after the day of first appearance of jaundice (acute and convalescent sera), but in none of 17 preillness sera collected from 4 to 25 days prior to the onset of jaundice. The temporal course of anti-HAV acquisition for the cases in this outbreak is illustrated in Fig. 4. The antibody curve again docu-

TABLE 3. *Coded serum panel 3: IEM and RIA for anti-HAV*

Code no.	Description	IEM[a]	RIA(P/N)[b]	Code no.	Description	IEM[a]	RIA(P/N)[b]
42230	Chimpanzee George 2 days post-HAV inoculation	−	− (0.9)	42240	Chimpanzee George 272 days post-HAV inoculation	+	+ (16.6)
42231	Chimpanzee George 13 days post-HAV inoculation	−	− (1.1)	42241	Chimpanzee S. preinoculation	−	+ (1.1)
42232	Chimpanzee George 30 days post-HAV inoculation[c]	+	+ (12.4)	39553	Chimpanzee G. preinoculation	−	− (1.5)
42233	Chimpanzee George 43 days post-HAV inoculation	+	+ (16.0)	36825	Experimental sheep antiserum	−	− (1.2)
42234	Chimpanzee George 57 days post-HAV inoculation	+	+ (20.3)	36826	Experimental sheep antiserum	−	− (2.0)
42235	Chimpanzee George 110 days post-HAV inoculation	+	+ (15.6)	36827	Experimental sheep antiserum	−	± (2.9)
42236	Chimpanzee George 139 days post-HAV inoculation	NT[d]	+ (14.7)	36828	Experimental goat antiserum	−	− (1.5)
42237	Chimpanzee George 174 days post-HAV inoculation	NT	+ (14.9)	42243	Recurrent familial cholestatic	−	− (0.8)
42238	Chimpanzee George 201 days post-HAV inoculation	+	+ (17.6)	42444	Jaundice	−	− (0.9)
42239	Chimpanzee George 232 days post-HAV inoculation	NT	+ (20.5)	42242	Human convalescent hepatitis A serum	+	+ (15.9)
				41668	Human convalescent hepatitis A serum	+	+ (5.4)
				41675	Human convalescent hepatitis A serum	+	+ (34.5)
				41678	Human convalescent hepatitis A serum	+	+ (4.6)

[a] Results kindly provided under code by Arie Zuckerman.

[b] *P/N* ratio computed from counts per minute of test serum divided by mean counts per minute of 10 negative sera. Sera were tested at a dilution of 1:1,000 with 30 *N* units of HAV (*N* = 138 cpm) per assay.

[c] SGPT activity elevated.

[d] NT, Not tested.

ments the early anti-HAV response in relation to the onset of icterus and is quite similar to that derived from the experimentally infected chimpanzees (Fig. 2).

RIA for IgM anti-HAV. Acute- and convalescent-phase sera from the human cases of naturally acquired hepatitis A (panel 4), as well as acute- and convalescent-phase sera from chimpanzees experimentally infected with HAV (panel 1), were examined for IgM anti-HAV by the RIA-blocking (RIA-MB) procedure described above. Table 5 summarizes the RIA-MB results for acute- and convalescent-phase sera, respectively.

Without exception, all 11 acute-phase sera were significantly blocked by goat antiserum to human IgM (Table 5). The maximum blocking effect was noted for an antiserum dilution of 1:500 and test serum dilutions of 1:10,000 to 1:100,000. Maximum RIA-blocking effects ranged between 59 and 95% (average of 78%), when compared with the matched NGS controls. It is interesting to note that one chimpanzee acute-phase IgM preparation (day 19 post-inoculation; SGPT peak, day 26) was almost entirely blocked by anti-IgM in the RIA-MB test. None of the 11 human or chimpanzee hepatitis A convalescent sera (long-standing antibody to HAV) was found to be blocked by anti-IgM in the RIA-MB test (Table 5). In fact, increases in specific counts per minute were noted for some anti-IgM-blocked test sera, when compared with their matched NGS controls. Convalescent chimpanzee IgG (serum collected 515 days after inoculation) at dilutions of 10^{-4} or 10^{-5} was also not blocked by anti-IgM.

Two other commercially available antisera to human IgM (Mu chain specific) were used in the RIA-MB procedure for repeat assay of selected acute- and convalescent-phase sera for IgM anti-HAV. These antisera (goat, Hyland; goat, Miles-Yeda) were found to react optimally in the RIA-MB procedure at a dilution of 1:1,000. All three antisera to IgM were screened for optimum blocking effects at dilutions of 1:100, 1:500, and 1:1,000.

TABLE 4. *Common-source outbreak of hepatitis A: summary of RIA anti-HAV results*

Type of serum	No. tested	No. of anti-HAV positive	Percent positive
Prejaundice	17	0	0
Acute phase (jaundice)	7	7	100
Convalescent phase	24	24	100

DISCUSSION

A marked reduction in serum reactivity in the RIA test for anti-HAV was noted for serum

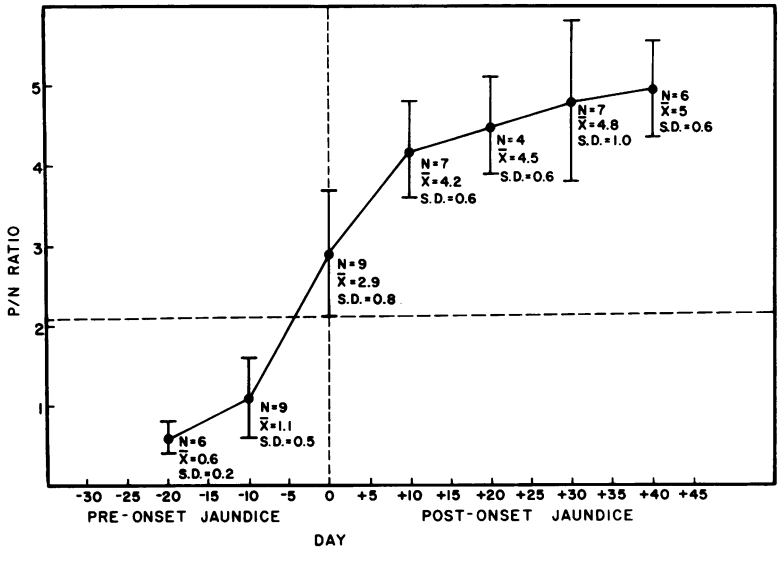

FIG. 4. *RIA for anti-HAV in preillness-, acute-illness (jaundice)-, and convalescent-phase sera from 24 icteric cases of hepatitis A. These sera were derived from the common-source outbreak described in the text. Five N units of HAV were used in each assay (N = 248 cpm). Dashed horizontal line represents 2.1 × N, used as the negative cut-off boundary. Dashed vertical line intersecting day 0 on the X-axis represents the day jaundice was first noted. Mean anti-HAV P/N ratios for each cluster of sera are shown bracketed by ±1 standard deviation (SD).*

TABLE 5. *RIA-MB for IgM anti-HAV in hepatitis A acute- and convalescent-phase human and chimpanzee sera*

Serum	$-\text{Log}_{10}$ dilution	RIA (cpm)[a]		Reduction[d] (%)	Serum	$-\text{Log}_{10}$ dilution	RIA (cpm)[a]		Reduction[d] (%)
		NGS[b]	GAS[c]				NGS[b]	GAS[c]	
Acute phase					Human 2	4	342	398	NR
CT 270	4	2,093	1,775	15	Human 2	5	50	85	NR
CT 270	5	1,653	217	87					
					Human 3	4	422	617	NR
CT 276	4	2,052	1,698	17	Human 3	5	0	42	NR
CT 276	5	214	46	79					
					Human 4	4	978	979	NR
CT 324	4	2,212	1,537	31	Human 4	5	50	77	NR
CT 324	5	128	53	59					
					Human 5	4	723	995	NR
CT 325	4	1,829	1,199	34	Human 5	5	58	96	NR
CT 325	5	1,496	537	64					
					Human 6	4	644	802	NR
CT 260	4	1,021	785	23	Human 7	4	455	497	NR
CT 260	5	273	25	91	Human 8	4	791	817	NR
CT 310	4	882	601	32	Chimp 1	4	1,620	1,581	2
CT 310	5	97	19	80	day 360	5	429	458	NR
CT 338	4	825	215	74	Chimp 2	4	819	805	2
CT 333	4	530	110	79	day 548	5	121	175	NR
CT 363	5	396	64	84					
Chimp 1	4	545	169	69	Chimp 3	4	757	953	NR
Chimp 2	4	106	29	73	day 921				
Chimp IgM[e]	4	1,344	66	95	Chimp IgG[g]	4	333	410	NR
Chimp IgM	5	131	28	79	day 515	5	114	145	NR
Convalescent phase									
Human 1	4	1,031	1,093	NR[f]					
Human 1	5	44	82	NR					

[a] Specific counts per minute. Fifteen *N* units of HAV were used per assay.
[b] NGS, Normal goat serum, 1:500.
[c] GAS, Goat antiserum to IgM (Behring), 1:500.
[d] In specific counts per minute versus NGS control.
[e] IgM fraction from acute-phase serum.
[f] NR, No reduction.
[g] IgG fraction prepared from serum collected 515 days after inoculation of chimpanzee with HAV.

dilutions lower than 1:1,000. One possible explanation for this phenomenon is the nonspecific desorption of test serum anti-HAV from the microtiter plate well during incubation with reagent HAV. The net effect would be a loss of antibody-bound HAV during the wash cycle. This loss of complexed HAV would result in a reduced binding of ^{125}I-labeled anti-HAV (IgG). Such an effect would be expected to be more pronounced at lower serum dilutions. As a consequence, all sera to be screened for anti-HAV by RIA were first diluted 1:1,000 in PBS.

A recent report by Dienstag et al. (4) compared the sensitivities of IEM, IAHA, and RIA for anti-HAV in late-convalescent-phase sera. RIA anti-HAV titers were lower than IAHA antibody titers, and IEM ratings did not correspond linearly with IAHA titers. Dienstag et al. concluded, based on sensitivity, specificity, and ease of application, that IAHA was the most appropriate serological procedure for evaluation of type A hepatitis. Our study, however, indicated that solid-phase RIA for anti-HAV was both highly specific and sensitive. RIA anti-HAV results agreed well with those obtained by IEM or IAHA. RIA, however, did detect acute-phase anti-HAV, whereas IAHA often did not. Analysis of human and chimpanzee preimmune-, acute-, and convalescent-phase sera by RIA for anti-HAV demonstrated the specificity of the direct RIA procedure. Sensitivity of the RIA procedure can be inferred from the anti-HAV-positive results obtained for test serum dilutions of 1:1,000,000 or greater. Detec-

tion of long-standing antibody to HAV in an adult urban population (11) also demonstrated the sensitivity of our RIA procedure for long-standing anti-HAV. Approximately 75% of the individuals over 50 years of age were found to be seropositive for anti-HAV. Sera from these individuals were screened for anti-HAV at a dilution of 1:1,000.

Examination of IgM and IgG fractions from an acute-phase chimpanzee serum sample by RIA revealed that IgM contained the bulk of the antibody specific for HAV. Although the relative avidity of either acute-phase IgM or IgG for HAV was not known, it appeared from the titration data that the IgG fraction contained less than 10% of the HAV binding capacity of the IgM fraction. This observation may help explain why both IEM and RIA detected anti-HAV in acute-phase sera, whereas IAHA often did not (13, 14). Our data suggest that IAHA detects convalescent-phase (IgG) anti-HAV, but does not readily detect acute-phase (IgM) anti-HAV.

Modification of the RIA procedure for anti-HAV enabled us to correctly identify acute- and convalescent-phase sera obtained from individuals with hepatitis A. Acute-phase sera, in which IgG anti-HAV had been diluted to extinction, or nearly to extinction, where shown to be specifically blocked by antisera to IgM. Convalescent-phase sera were not blocked by anti-IgM in the RIA-MB test. These findings support our observation that the primary acute-phase immunoglobulin is IgM. We presume that binding of anti-IgM to the Mu (heavy) chain of IgM blocked binding of HAV by altering the stereoconformation, or the accessibility, of the antigen-combining site on IgM. The ability of the RIA-MB procedure to detect IgM anti-HAV was influenced by at least three test variables, including: (i) the relationship of the serum collection date to the date of onset of clinical illness or jaundice; (ii) the dilution of the test serum; and (iii) the specificity and potency (blocking effect) of the goat antiserum to human IgM. Our preliminary data suggest that test sera collected between the onset of clinical illness and the appearance of jaundice react optimally in the RIA-MB procedure. Confirmation of this, however, must await the analysis by RIA-MB of larger numbers of acute-phase sera.

Prior to the development of the RIA-MB procedure reported here for IgM anti-HAV, two sera were required for the diagnosis of hepatitis A by IEM, IAHA, RIA, or complement fixation. In addition, the IAHA and complement fixation test procedures required titration of the paired acute- and convalescent-phase sera. The previously described RIA procedures (7, 16) required the acquisition of paired preimmune- and convalescent-phase sera for the diagnosis of hepatitis A. The RIA-MB procedure described in this report required only a single acute-illness phase serum specimen for the diagnosis of viral hepatitis A. Our experience indicated that two dilutions of a test serum and a single optimum dilution of a standard goat antiserum to human IgM were usually adequate for the detection of acute-phase IgM anti-HAV. Another advantage of the RIA-MB procedure was its use of limited quantities of purified HAV. The RIA-MB procedure appears to be a potentially useful serological tool for the diagnosis of viral hepatitis A in the acutely ill patient, since the majority of acute-phase antibody is IgM. The early diagnosis of acute hepatitis A, particularly in suspected common-source outbreaks, should significantly improve our ability to control this viral disease.

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