JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1979, p. 120-127
0095-1137/79/01-0120/08$02.00/0

Vol. 9, No. 1

Serodiagnosis of Viral Hepatitis A by a Modified Competitive Binding Radioimmunoassay for Immunoglobulin M Anti-Hepatitis A Virus

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Received for publication 14 September 1978

A competitive binding radioimmunoassay (CBA) for antibody to hepatitis A virus (HAV) was evaluated and compared with a standard solid-phase radioimmunoassay for anti-HAV. CBA was found to be sensitive and specific for the detection of anti-HAV, as demonstrated by the 98% concordance of CBA and solid-phase radioimmunoassay test results. The standard CBA test was modified for the differential detection of acute (immunoglobulin M) and convalescent (immunoglobulin G) anti-HAV by incorporation of a step in which immunoglobulin G anti-HAV was preferentially absorbed with *S. aureus* cells (protein A). The modified CBA test was shown to be capable of differentiating between acute- and convalescent-phase sera. The modified CBAM test was able to detect immunoglobulin M anti-HAV up to approximately 4 weeks after the onset of illness.

Significant progress in the understanding of viral hepatitis A (HAV) has been achieved through the development and application of "new" immunological techniques for the detection of both HAV and anti-HAV (1-4, 7, 9, 12, 13, 17-19). Anti-HAV can be detected by immune electron microscopy (6, 8), complement fixation (15), immune adherence hemagglutination (10, 14), and by micro-solid-phase radioimmunoassay (SPRIA) procedures (11, 16). Unfortunately, detection of anti-HAV by immune electron microscopy is a cumbersome and time-consuming technique that demands the skills of an experienced electron microscopist, whereas complement fixation is a relatively insensitive technique for the detection of anti-HAV and requires comparatively large quantities of reagent HAV (antigen). On the other hand, immune adherence hemagglutination is a very sensitive technique for detecting anti-HAV but it, too, requires large amounts of reagent HAV. In view of these restrictions, RIA appears to be, at present, the most suitable technique for screening large numbers of sera for anti-HAV, since it is both sensitive and specific and requires relatively small quantities of reagent HAV (5).

In contrast to immune adherence hemagglutination, which does not detect early (acute) anti-HAV (4), standard RIA procedures detect both immunoglobulin M (IgM) and IgG anti-HAV and do not, therefore, differentiate between acute (IgM) and convalescent or preexisting (IgG) antibody. A modified RIA procedure was recently described (5) in which IgM anti-HAV was specifically detected by incorporation of a step in which IgM anti-HAV was blocked with goat anti-IgM (μ chain specific). This latter experimental procedure was shown to be useful in the serodiagnosis of acute viral hepatitis A, however, large-scale application of this type of test awaited the commercial production of an RIA procedure that could be readily adapted for the differential detection of IgM and IgG anti-HAV. We now describe a competitive binding RIA for anti-HAV that, in a modified form, is suitable for the serodiagnosis of acute HAV. Data are also presented that demonstrate the concordance of the standard competitive binding RIA (CBA) with an established double-antibody sandwich RIA for anti-HAV.

MATERIALS AND METHODS

RIA for antibody to HAV: SPRIA. SPRIA for anti-HAV in serum was performed as previously described (5). Samples of serum were diluted 1:1000 before testing. All sera were run in duplicate.

CBA. The CBA procedure for anti-HAV (HAVAB; Abbott Laboratories, Inc. is based on the principle of competitive binding of serum anti-HAV with ^{125}I-labeled IgG anti-HAV to solid-phase HAV antigen. A brief description of the CBA test follows. In one of the wells of a 20-well reaction tray, 200 μl of [^{125}I]IgG anti-

HAV are mixed with 10 μl of undiluted patient serum. Next, a polystyrene bead, on which HAV Ag has been bound, is added. (Polystyrene beads were coated with HAV by immersion in a beaker containing a liquid suspension of an HAV-positive primate liver pool. The beads were then removed from the suspension, air dried at room temperature, and stored at 4 to 8°C until used). The reaction tray is then covered with an adhesive sheet, gently agitated, and incubated for 20 h at room temperature (25 to 26°C). The reaction mixture is aspirated, and the bead is washed with two 5-ml portions of deionized water by using a Pentawash II system, or the equivalent. The bead is transferred to a counting tube and counted in a gamma counter for 1 min.

The validity of the CBA test is evaluated by comparing the net mean count rate of seven negative sera to that of four positive sera. More specifically, net negative control counts per minute (cpm's) ($NC\bar{x}$) divided by the net positive control cpm's ($PC\bar{x}$) is defined as the N/P ratio. The N/P ratio for any given test kit must be greater than 5.0 to ensure the validity of the test results.

Specimen results are evaluated by determining a cpm cutoff value, defined here as *D*. The *D* value is calculated by dividing the sum of $NC\bar{x}$ (normally around 20,000 cpm) and $PC\bar{x}$ by 2. A "gray zone" is calculated by bracketing the cutoff value by 10% (0.9 *D* to 1.1 *D*). Any test serum with a cpm less than 0.9 *D* (ca. 50% competition) is considered positive for anti-HAV, whereas any with a cpm greater than 1.1 *D* is considered negative. Sera yielding cpm values within the range of 0.9 *D* to 1. *D* are retested. All CBA results are expressed as percent competition, or simply as positive or negative.

CBAM. The standard CBA procedure was modified (CBAM) for the differential detection of IgM and IgG anti-HAV in serum by incorporation of a step in which IgG anti-HAV was preferentially absorbed with *Staphylococcus aureus* cells positive for protein A (referred to hereafter as protein A). A 1-g amount of dried staphylococcal cells (Newman D_2C strain from Sigma Chemical Co., St. Louis, Mo.) is suspended in 10.0 ml of 0.01 M phosphate-buffered saline (pH 7.2) containing 1:4,000 sodium azide. Volumes (100 μl each) of the suspension are pipetted into 1.0-ml microcentrifuge tubes (Sherwood Medical Industries, Inc., St. Louis, Mo.), one portion per serum dilution. The cell suspensions are centrifuged for 1 min at top speed in an International microcapillary centrifuge, model MB (International Equipment Co., Needham, Mass.); the supernatant fluids are discarded and the cell pellets are used as described below. Each serum to be tested is diluted in 0.01 M phosphate-buffered saline, pH 7.2 (containing 1:4,000 sodium azide) to a final volume of 150 μl; the volume of serum added depends on the actual cpm's of the sample in the standard CBA test. Sera with cpm's in excess of 4,000 are tested by CBAM at a final dilution of 1:5, whereas sera with cpm's less than 4,000 are tested at final dilutions of 1:20 or 1:30 (data not shown). A 100-μl amount of diluted serum is added to the cell pellet, mixed, and incubated for 30 min at room temperature. After centrifugation to pellet the cells, two 10-μl samples of the supernatant (absorbed serum) are transferred to a reaction tray for analysis by the standard CBA procedure. Two 10-μl samples of the original serum dilution (unabsorbed control serum) are simultaneously tested by CBA to yield control cpm's. For the CBAM test to be valid, the diluted control serum must be positive by the standard CBA procedure. Diluted control sera negative for anti-HAV by the CBA procedure must be retested at a lower dilution. The antibody type of an anti-HAV-positive serum can also be evaluated by computing the ratio (*R*) of sample cpm's before (−A) and after (+A) protein A absorption ($R = +A/-A$). Analysis by CBAM of sera from cases of viral hepatitis A (data shown below) revealed that *R* was less than 2.5 for all acute-illness phase sera and was equal to or greater than 2.5 for all convalescent-phase sera (Tables 4 and 5). Also, any serum with an *R* value less than 2.5 was judged to be positive primarily for IgM anti-HAV, whereas any serum with an *R* value equal to or greater than 2.5 was considered to be positive primarily for IgG anti-HAV. On the basis of our findings, we concluded that the *R* value of 2.5 adequately discriminated between acute- and convalescent-phase serum samples and, therefore, was chosen as the cutoff point.

Serum panels. panel 1: human and chimpanzee sera. A total of 110 sera were obtained from serologically confirmed cases of viral hepatitis A or B, from individuals negative for anti-HAV, and from chimpanzees experimentally infected with HAV at the Hepatitis Laboratories Division (HLD). All of these sera had been previously tested at the HLD by SPRIA for anti-HAV.

Panel 2: first-time blood donors. A total of 152 sera from first-time volunteer blood donors in North Dakota were screened by CBA for anti-HAV. Of these sera, only three (2.0%) were positive for anti-HAV. The prevalence of anti-HAV in this population was judged to be extraordinarily low; therefore, a subset of 80 sera negative for anti-HAV was selected for retesting by SPRIA to evaluate any possible false-negative results by the CBA procedure.

Panel 3: serial chimpanzee specimens. Serial serum specimens from two chimpanzees (numbers 0770 and 0084) experimentally infected with HAV (1) were obtained from the day of inoculation (day 0) through day 66 after inoculation for animal no. 0770, and through day 79 after inoculation for animal no. 0084. Late convalescent-phase sera were obtained from animals no. 0770 and 0084 on days 531 and 830, respectively, after inoculation. Additional sera from three other HAV-infected chimpanzees were obtained from the serum bank of the HLD. All of these sera were from enzymatically, histopathologically, and serologically confirmed cases of HAV.

Panel 4: acute and convalescent sera. A total of 22 sera (18 human, 4 chimpanzee) containing long-standing (convalescent) antibody to HAV were obtained from the HLD serum bank. These sera were from cases of HAV that had occurred at least 1 year before the collection of the convalescent serum. Twenty sera (12 human, 8 chimpanzee) containing acute-phase anti-HAV were obtained from clinically and serologically confirmed cases of HAV as well as from chimpanzees experimentally infected with HAV at the HLD. All human sera were collected within 3

days of the onset of clinical illness, and all chimpanzee sera were obtained within the interval of 7 days before and after the peak of serum glutamic pyruvic transaminase (SGPT) activity.

Panel 5: common-source outbreak. Sera from 62 individuals involved in a common-source outbreak of HAV that occurred in a small village in France in 1976–1977 were kindly provided by Michele Aymard of the Laboratoire de Virologie, Universite Claude Bernard, Lyon, France. A total of 15 sera were collected within 2 days of the onset of symptoms, whereas the remaining sera were collected at varying intervals of time up to 31 days after the onset of symptoms. All patients presented with clinical and biochemical evidence consistent with a diagnosis of viral hepatitis, subsequently confirmed to be type A by serological examination, as reported in this paper.

RESULTS

The specificity and sensitivity of the CBA procedure were evaluated by tested coded panels of human and chimpanzee sera that had previously been examined for anti-HAV by an established SPRIA procedure or by immune electron microscopy. The specificity and sensitivity of the SPRIA test have been previously documented (5). Reanalysis of serum panel 1 by CBA and SPRIA (Table 1) showed 56% ($n = 62$) to be positive for anti-HAV by CBA, whereas 54% ($n = 59$) were found positive by SPRIA. Of 110 total sera tested by CBA and SPRIA, 3 gave discordant results. The three discordant test results were considered to be false negatives by SPRIA, since all three sera were obtained from serologically confirmed cases of viral hepatitis A. Analysis of a subset of 80 sera from a study of first-time volunteer blood donors (panel 2) revealed all 80 to be negative for anti-HAV by CBA, whereas SPRIA detected antibody in one serum specimen (P/N = 4.3).

Serial serum specimens from two chimpanzees experimentally infected with HAV (panel 3) were tested by CBA and SPRIA for anti-HAV (Table 2). Anti-HAV was detected in chimpanzee no. 0770 serum by CBA and SPRIA as early as 17 days after inoculation. The ability of sera to competitively block binding of ^{125}I-labeled IgG anti-HAV in the CBA procedure increased from 73 to 91% between 17 and 66 days after inoculation. The SPRIA P/N ratio increased from 11.4 to as high as 26.0 (day 56) during the same interval of time. SPRIA detected anti-HAV in serum from chimpanzee no. 0084 as early as 10 days after inoculation (P/N = 2.2), whereas sera tested by CBA were first found to be positive on day 17. The ability of chimpanzee no. 0084 sera to competitively block ^{125}I-IgG anti-HAV increased from 78 to 94% between 17 and 79 days after inoculation. The SPRIA P/N ratio was found to increase from 2.2 to as high as 41.7 (day 35) between 10 and 79 days after inoculation.

Sera from chimpanzees no. 0770 and 0084 were also run by the CBAM procedure to differentially detect IgG and IgM anti-HAV, as described above. Sera obtained from animal no. 0770 between 0 and 531 days after inoculation were examined for anti-HAV at a dilution of 1:30 (Fig. 1). Sera from days 17 through 531 were found to be positive by the standard CBA procedure, whereas only sera collected between 17 and 52 days after inoculation were shown to be positive in the CBAM test (i.e., after absorption with protein A). The ratio of the test cpm's of a serum sample absorbed with (+A) or without (−A) protein A, defined as $R = +A/-A$, was found to range between approximately 1.1 and

TABLE 1. *CBA and SPRIA for anti-HAV in chimpanzee (n = 39) and human (n = 71) sera*

Procedure	Sera[a]	
	Positive	Negative
CBA	62 (56)	48 (44)
SPRIA	59 (54)	51 (46)

[a] Values indicate number of sera and, in parentheses, percentage of total sera.

TABLE 2. *Development of anti-HAV in two experimentally infected chimpanzees: CBA versus SPRIA*

Day after inoculation	Chimp no. 0770		Chimp no. 0084	
	CBA % competition[a]	SPRIA P/N ratio[b]	CBA % competition[a]	SPRIA P/N ratio[b]
0	31	1.1	0	0.8
3	21	0.8	0	1.0
7	20	1.1	2	1.3
10	15	1.1	7	2.2
14	28	1.7	0	16.2
17	73	11.4	78	35.5
21	81	13.2	88	34.0
24	87	17.5	87	30.0
28	89	17.4	90	38.1
35	91	20.5	92	41.7
38	91	21.0	94	37.5
42	90	25.0	94	38.5
45	90	20.7	94	38.6
49	91	21.0	94	41.0
52	90	20.4	94	31.9
56	91	26.0	95	31.1
59	91	24.7	95	29.4
63	NT[c]	NT	94	29.4
66	91	20.2	94	31.3
79	NT	NT	94	38.4

[a] Values indicate percent competition versus negative control.

[b] P/N ratio $\geq$ 2.1 indicates serum positive for anti-HAV.

[c] NT, Not tested.

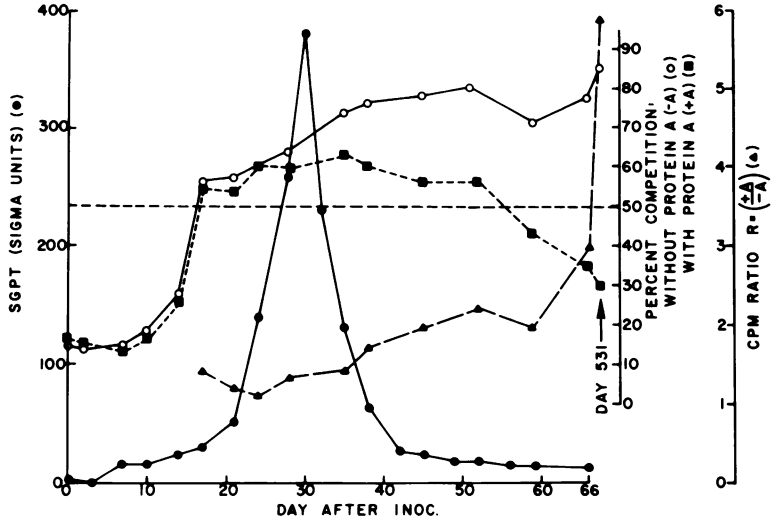

FIG. 1. *Development of anti-HAV in a chimpanzee (no. 0770) experimentally infected with HAV. Anti-HAV activity in serial serum specimens was determined by the CBA and CBAM procedures described in the text.*

1.4 on days 17 to 24 to as high as 5.9 on day 531 after inoculation. *R* was found to be less than 2.5 from day 17 through day 59 after inoculation, or as long as 29 days past the peak of SGPT activity.

Sera obtained from animal no. 0084 between 0 and 830 days after inoculation were also examined by CBAM at a dilution of 1:30 (Fig. 2). Sera from days 21 through 830 were shown to be positive for anti-HAV by the standard CBA procedure, whereas only sera obtained between days 21 and 38 after inoculation were found to be positive following absorption with protein A. The ratio *R* (defined above) showed an increase from approximately 1.0 on day 17 to 7.8 on day 830 (Fig. 2). The value of *R* was less than 2.5 up to at least 42 days after inoculation, or as long as 11 days past the peak of SGPT activity.

The ability of the CBAM procedure to differentially detect IgM or IgG anti-HAV was documented by analyzing partially purified IgM and IgG from acute- and early convalescent-phase sera obtained from subjects with hepatitis A. Serum specimens were fractionated by ion-exchange chromatography (diethylaminoethylcellulose), and pools of the IgM- and IgG-rich fractions were titered by CBA and then tested by CBAM. Table 3 shows the results for sera obtained from one subject 4 and 64 days after the onset of jaundice. These results are representative of findings for acute and early convalescent serum pairs obtained from other cases of hepatitis A. The acute and early convalescent sera had titers of 1:30 and 1:150, respectively. Titers of the pooled IgM and IgG fractions showed that in acute-phase serum anti-HAV activity was stronger in the IgM fraction than in the IgG fraction, whereas in the early convalescent serum anti-HAV was predominantly in the IgG fraction. However, some anti-HAV was also present in the IgM fraction of the latter serum, a finding consistent with the notion that early convalescent serum contains both IgM and IgG anti-HAV. When the day 4 and day 64 pools of antibody were absorbed with protein A, little of the IgM anti-HAV activity was absorbed, whereas most of the IgG anti-HAV activity was absorbed. The residual anti-HAV activity in the day 4 serum (after protein A absorption) was found only in the IgM fraction. Although the day 64 serum remained positive for anti-HAV after protein A absorption, the *R* value of 3.7 suggested that the majority of anti-HAV activity was in the IgG fraction. In fact, the titer of the day 64 IgG pool was 1:32 compared to 1:4 for the IgM pool. These data demonstrate that anti-HAV activity in acute-phase serum is contained primarily in the IgM fraction, whereas in early convalescent-phase serum it is contained primarily in the IgG fraction. Convalescent sera collected more than 4 months after the resolution of disease contain anti-HAV activity that is detectable primarily, if not completely, in the IgG fraction (data not shown). Absorption of these sera with protein A results in the loss of anti-HAV activity; *R* values are usually found to exceed 4.0 (Table 4).

Convalescent-phase sera containing long-standing antibody against HAV (panel 4) were examined by the CBAM procedure at dilutions of either 1:5 or 1:30, as described above. All sera were shown to contain antibody that could be

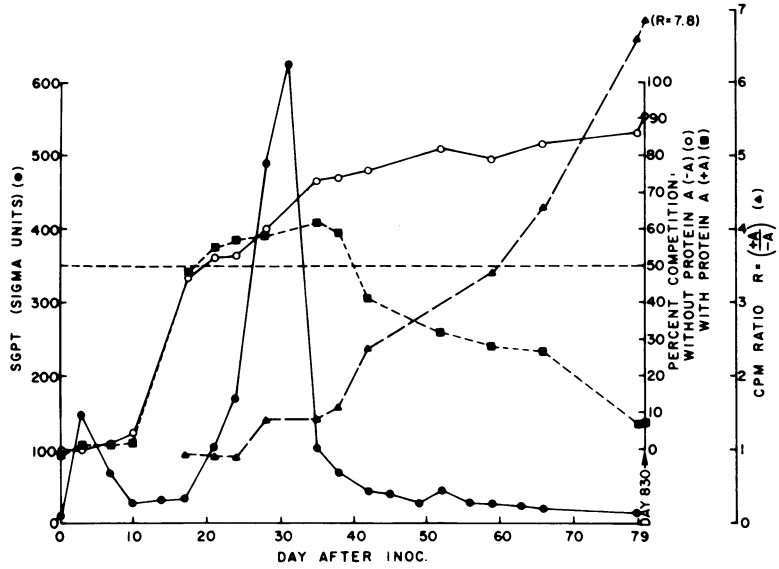

FIG. 2. *Development of anti-HAV in a chimpanzee (no. 0084) experimentally infected with HAV.*

TABLE 3. *Titer (CBA) and CBAM analysis for anti-HAV in IgG and IgM fractions from acute- and early convalescent-phase sera*

Specimen	Serum			IgG Pool[a]			IgM Pool[a]		
	cpm	Titer	R^b	cpm	Titer	R	cpm	Titer	R
Day 4									
Unabsorbed	5,852[c]	1:30		11,376	1:4		8,678	1:8	
			1.7			—[d]			—
Absorbed	10,027			21,245			10,235		
Day 64									
Unabsorbed	1,432[c]	1:150		1,854	1:32		7,727	1:4	
			3.7			6.3			—
Absorbed	5,222			11,727			9,633		

[a] Tested undiluted. Net negative control cpm ($NC\bar{x}$) = 25,767; net positive control cpm ($PC\bar{x}$) = 805; cpm cutoff value (D) = 13,286.

[b] See text for definition of R.

[c] Tested at 1:10 dilution.

[d] —, cpm value of unabsorbed pool did not meet criteria for calculation of R values.

absorbed with protein A (Table 4). In no case was a serum still positive for anti-HAV after absorption with protein A. The ratio R = +A/−A ranged from 2.71 to 8.82 ($\bar{x}$ = 4.96; standard deviation [SD] = 1.57). R values for sera run at a dilution of 1:5 were statistically not significantly different from R values for sera run at a dilution of 1:30. Sera obtained from acute ill individuals and from chimpanzees in the acute-phase of hepatitis A were also tested by the CBAM procedure (Table 5). All of these sera contained anti-HAV activity that could not be absorbed with protein A. The ratio R ranged from 0.70 to 1.60 ($\bar{x}$ = 1.21; SD = 0.22). Statistical analysis of the mean R values for the convalescent and acute sera showed them to be significantly different (t = 11.33; $P < 0.0001$). The lowest R value for a convalescent serum (Table 3, human no. 8; R = 2.71) was approximately 7 SD units (SDU) above the mean R value for acute-phase sera, and was five SDU above the highest acute-phase R value (Table 5). The CBAM procedure was also used to screen sera obtained from individuals involved in a suspected outbreak of viral hepatitis A. Initial screening of sera from panel 5 by the standard CBA procedure showed all 62 samples to be positive for anti-HAV (data not shown). Retesting of 56 of these sera by the CBAM procedure showed that 55 (98%) contained anti-HAV that could not be absorbed with protein A. All of these sera were collected between 0 and 31 days

TABLE 4. *Differential RIA (CBAM) of sera containing long-term (convalescent) anti-HAV*

Serum no.	Dilution	CBAM[a]		R^b
		−Protein A	+Protein A	
Human 1	1:5	+	−	7.03
Human 2	1:5	+	−	7.51
Human 3	1:5	+	−	4.24
Human 4	1:5	+	−	5.08
Human 5	1:5	+	−	5.79
Human 6	1:5	+	−	7.12
Human 7	1:5	+	−	5.53
Human 8	1:5	+	−	2.71
Human 9	1:5	+	−	3.19
Human 10	1:5	+	−	2.84
Human 11	1:5	+	−	2.92
Human 12	1:5	+	−	4.81
Human 13	1:5	+	−	2.78
Human 14	1:5	+	−	5.21
Human 15	1:30	+	−	6.18
Human 16	1:30	+	−	3.64
Human 17	1:30	+	−	3.93
Human 18	1:30	+	−	5.93
Chimp 1	1:30	+	−	5.09
Chimp 2	1:30	+	−	7.82
Chimp 3	1:30	+	−	4.61
Chimp 4	1:30	+	−	5.69

[a] Results expressed as positive (+) or negative (−).
[b] See text for definition of R. $n = 22$; $\bar{x} = 4.96$; SD = 1.57.

after the onset of clinical illness. Of 41 sera collected between 0 and 21 days after the onset of illness, all were still found to be positive for anti-HAV after absorption with protein A, as were 14 (93%) of the 15 remaining sera collected between 22 and 31 days after the onset of illness. R values for sera collected up to 14 days after onset of illness ($n = 30$) ranged between 0.70 and 2.39 ($\bar{x} = 1.40$; SD = 0.45), whereas the R values for sera collected between 15 and 31 days after the onset of illness ($n = 26$) ranged between 0.91 and 14.9 ($\bar{x} = 2.53$; SD = 2.60). A t test for the significance of difference between the mean R value of sera collected between 0 to 14 days and 15 to 31 days after the onset of illness showed $t = 2.21$ (df = 55) or $0.02 < P < 0.05$.

DISCUSSION

The CBA procedure was shown to be a sensitive and specific test for anti-HAV. Of 110 sera examined by CBA and SPRIA (panel 1), 3 (2.7%) gave discordant results. Since these sera were obtained from previously confirmed cases of hepatitis A, the negative SPRIA test results were considered to be false negatives. It is of interest to note that analysis by CBA and SPRIA of a subset of 80 sera from a study of first-time volunteer blood donors showed a 99% concordance of test results. All 80 sera were negative for anti-HAV by CBA, whereas one serum was found to contain weakly reactive anti-HAV by SPRIA.

Sensitivity of the CBA test for anti-HAV was demonstrated by its ability to detect early acute-phase antibody in sera obtained from two chimpanzees experimentally infected with HAV (panel 3) (Table 2). In summary, of the combined sera (panels 1, 2, and 3) tested by CBA and SPRIA, 90 (39.5%) were found to be positive by CBA, whereas 89 (39.0%) were shown to be positive by SPRIA. Five sera (2.2%) gave discordant results in the CBA and SPRIA tests for anti-HAV. In addition to demonstrating the sensitivity and specificity of the CBA procedure, our test results also show that CBA detects IgM (early acute-phase) anti-HAV as demonstrated by the finding of anti-HAV in early acute-phase chimpanzee sera (Table 2). We have previously demonstrated that RIA procedures detect IgM as well as IgG anti-HAV infected chimpanzees, and that early acute-phase anti-HAV is primarily IgM antibody (5).

The ability of the CBAM test to differentiate between acute (IgM) and convalescent (IgG) anti-HAV was verified in part by examination of serum panels 3 and 4, as well as by examination of fractionated acute- and convalescent-phase sera. Sera obtained between 13 days before and up to 22 days after the peak of SGPT activity in chimpanzee no. 0770 were shown to contain anti-

TABLE 5. *Differential RIA (CBAM) of acute-illness sera containing anti-HAV*

Serum no.	Dilution	CBAM[a]		R^b
		−Protein A	+Protein A	
Human 1	1:30	+	+	1.06
Human 2	1:30	+	+	1.27
Human 3	1:30	+	+	1.37
Human 4	1:30	+	+	1.27
Human 5	1:30	+	+	1.28
Human 6	1:30	+	+	1.12
Human 7	1:30	+	+	0.70
Human 8	1:30	+	+	1.53
Human 9	1:30	+	+	1.02
Human 10	1:30	+	+	1.10
Human 11	1:30	+	+	1.10
Human 12	1:30	+	+	0.97
Chimp 1	1:30	+	+	0.92
Chimp 2	1:30	+	+	1.40
Chimp 3	1:30	+	+	1.41
Chimp 4	1:30	+	+	1.60
Chimp 5	1:30	+	+	1.22
Chimp 6	1:30	+	+	1.12
Chimp 7	1:30	+	+	1.32
Chimp 8	1:30	+	+	1.43

[a] Results expressed as positive (+) or negative (−).
[b] See text for definition of R. $n = 20$; $\bar{x} = 1.21$; SD = 0.22.

HAV activity that could not be absorbed with protein A, whereas the *R* value was found to be less than 2.5 for sera collected up to 29 days past the SGPT peak. The latter observation is of particular significance in that even though the serum specimen collected on day 59 was negative for anti-HAV in the CBAM test, the ratio *R* = +A/−A was still less than 2.5. In this case, the use of the ratio *R* extended by 7 days the ability of the CBAM test to detect a recent HAV infection. For chimpanzee no. 0084, sera obtained between 10 days before and 7 days after the peak of SGPT activity were shown to contain anti-HAV activity after protein A absorption, whereas the *R* value was found to be less than 2.5 up to 11 days past the SGPT peak. Here, as in the case of chimpanzee no. 0770, the use of the ratio *R* extended the ability of the CBAM test to detect a recent HAV infection.

Examination by CBAM of 18 human and 4 chimpanzee sera containing convalescent anti-HAV (panel 4) showed that all contained antibody that could be absorbed with protein A and that no serum had an associated *R* value less than 2.71. Examination by CBAM of 12 human and 8 chimpanzee acute-phase sera, on the other hand, revealed the presence of anti-HAV activity that could not be absorbed with protein A and that no serum had an associated *R* value greater than 1.60. These combined findings and the data presented in Table 3 demonstrate the ability of the CBAM test to clearly distinguish between acute and convalescent (preexisting) antibody to HAV.

Use of the CBAM test to screen sera from a suspected outbreak of HAV in France further demonstrated the ability of the procedure to identify bona fide cases of acute hepatitis A. Examination by CBAM of acute-phase sera obtained during this outbreak showed that 98% contained anti-HAV activity that could not be absorbed with protein A and that all of these had *R* values less than 2.5. These results confirmed that the outbreak was due to active infection by HAV. Sera collected beyond 14 days after onset of symptoms tended to have increasing *R* values, as would be expected for sera containing increasing titers of IgG anti-HAV and decreasing titers of IgM anti-HAV. Only one serum collected between 0 and 31 days after onset of illness was shown by CBAM to be negative for anti-HAV after absorption with protein A.

Our experience with the CBAM test has shown that sera run undiluted may not yield valid results, presumably because all of the IgG anti-HAV cannot be absorbed with protein A. We have also found that diluted sera (unabsorbed controls) that yield cpm's substantially greater than one-half the cutoff value in the CBA test may give lower than expected *R* values. Preliminary results (data not shown) suggest these sera should be retested at a lower dilution (but no lower than 1:2) to achieve consistent results.

Development of the CBAM test now makes it possible to diagnose cases of hepatitis A in the acute phase of illness by using only a single serum specimen. In combination with RIAs for HBsAg, anti-HBc, and anti-HBs, the CBAM test will also allow for the etiological differentiation of cases of viral hepatitis, thus providing a valuable tool for the study of hepatitis due to other (non-A, non-B) hepatitis viruses.

ADDENDUM IN PROOF

Since the initial writing of this paper, we have found that some lots of the HAVAB kit do not have $NC\bar{x}$ values similar to that of the kits used in our original study (20,000 to 30,000 cpm). Such kits, in our experience, may require the user to make serum dilutions that are different from those described in our paper, particularly if $NC\bar{x}$ is substantially less than 20,000 cpm. For example, we have found that in kits with $NC\bar{x}$ = 9,000 cpm, sera giving 90% or greater competition (900 cpm) in the HAVAB test should be diluted 1:15 for protein A absorption, whereas sera giving less than 90% competition should be diluted 1:2 or 1:5. With these kits, an *R* value of 2.2 appears to adequately discriminate between IgG (preexisting) and IgM (acute-phase) anti-HAV. Additional experience with the modified HAVAB procedure (CBAM) has also shown that the *R* value is a more reliable criterion for the determination of the anti-HAV antibody class than is the positivity or negativity of the diluted serum after protein A absorption.

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