Enzyme-Linked Immunosorbent Assay for Detection of Hepatitis A Antigen in Stool and Antibody to Hepatitis A Antigen in Sera: Comparison with Solid-Phase Radioimmunoassay, Immune Electron Microscopy, and Immune Adherence Hemagglutination Assay

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Previously described techniques for detection of hepatitis A antigen (HA Ag) and antibody (anti-HA) have required purified HA Ag and expensive equipment. Herein is described an enzyme-linked immunosorbent assay (ELISA) for specific detection of HA Ag in human stool filtrates and of anti-HA in sera by using selected HA Ag-containing human stool filtrates as the antigen source. Because human stools often react nonspecifically in serological tests for HA Ag, blocking with preexposure and hyperimmune anti-HA sera from a chimpanzee inoculated with hepatitis A virus was used to confirm specific detection of HA Ag. The sensitivity of ELISA was found to be comparable to that of solid-phase radioimmunoassay (SPRIA) and immune electron microscopy (IEM). Of 37 acute-phase stools collected from nine patients, 16 were positive for HA Ag by ELISA. In 13 of these, HA Ag particles were found by IEM, and an additional 3 stools negative by ELISA contained HA Ag particles by IEM. Eight control stools were negative by both ELISA and IEM. Anti-HA was measured in sera by demonstrating its ability to block binding of the enzyme conjugate to HA Ag in a stool without detectable nonspecificity. This test (blocking ELISA) was as sensitive and specific as blocking SPIRA, IEM, and immune adherence hemagglutination and, like SPRIA and IEM, detected early-developing antibody. The ELISA is simple to perform and requires only a minimum of equipment. It is useful for screening stools for HA Ag and for monitoring HA Ag during purification, as well as for detecting early and late anti-HA in sera.

Since the finding in 1973 by Feinstone et al. of 27-nm virus-like particles by immune electron microscopy (IEM) in stools from humans with acute hepatitis A virus infection (9), several new techniques have been used to detect this hepatitis A antigen (HA Ag) and its antibody (anti-HA) (10-12, 14-16, 18, 20). Immune adherence hemagglutination (IAHA) is now the most commonly used test for detection of anti-HA, but it requires purified HA Ag either from marmoset (*Saguinus mystax*) liver (15) or from human (16) or chimpanzee stool (unpublished data).

Purcell et al. and Hollinger et al. developed solid-phase radioimmunoassays (SPRIA) for detection of HA Ag (12, 20). These tests were useful for the detection of HA Ag in small vol-

umes and could more easily be applied to a larger number of samples than IEM. They proved to be very useful in monitoring the progress of purification attempts. These techniques also were used to detect HA Ag in human stool suspensions, but, due to a high frequency of false positive results, the tests were considered unreliable for this purpose. Recently Hall et al. reported the detection of HA Ag in 5% stool extracts by SPRIA; they found the test sensitive but again not very specific for detecting unpurified HA Ag (10).

In 1971, Engvall and Perlmann (8) and Van Weemen and Schuur (23) described an enzyme-like immunosorbent assay (ELISA) for detection of immunoglobulin G (IgG) and human chorionic gonadotropin. The test is very similar to radioimmunoassay (RIA) but uses an enzyme as label instead of an isotope. The test has several

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advantages over RIA. The conjugate is stable for months and probably years, no expensive counting equipment is necessary, and there is no radiation safety hazard. Several studies have found the ELISA and RIA to be of equal sensitivity (23a, 25, 26). We describe here an ELISA technique for the specific detection of HA Ag in 2% stool suspensions and the detection of anti-HA in sera by its ability to inhibit (block) the binding of the enzyme conjugate. These tests use purified stool suspensions as antigen and make possible the detection of HA Ag and anti-HA without expensive laboratory equipment.

MATERIALS AND METHODS

Reagents. Stools were collected during the acute phase of illness from Navy recruits involved in an outbreak of hepatitis type A at the San Diego Naval Training Center, and, from eight patients, a second stool was collected 3 months later (7). Daily stools were collected from four chimpanzees experimentally infected with the MS-1 strain of hepatitis A virus (5). Twenty percent stool extracts collected from humans during experimental infection with MS-1 virus were also studied (2, 4). Two percent stool filtrates were prepared as previously described (5). All stools were stored at -70°C , usually as 2% filtrates, for several years.

Sera. Serial sera obtained from humans contracting hepatitis type A during the San Diego outbreak as well as from chimpanzees and humans experimentally infected with hepatitis A virus were tested for anti-HA. A convalescent serum from a patient (P) with naturally occurring hepatitis A virus infection 3 months previously (a family outbreak) and a hyperimmune anti-HA chimpanzee serum (6) were used for enzyme and ^{125}I conjugation, and a convalescent serum (5) from a human (S) who contracted hepatitis during the San Diego outbreak was used for ^{125}I conjugation. Convalescent sera from two children (no. 60 and 67) contracting hepatitis type A during the Greenland epidemic in 1970–1974 (22) and the P serum were evaluated for precoating the microtiter plates. Preinoculation serum (presera) from a chimpanzee developing hepatitis type A after experimental inoculation with hepatitis A virus and the hyperimmune serum taken after boosting the same chimpanzee with purified HA Ag were used in a 10^{-2} dilution for specificity testing in ELISA.

Conjugates. IgG was isolated from the P and hyperimmune sera by ammonium sulfate precipitation and diethylaminoethyl-cellulose column chromatography and labeled with horseradish peroxidase (HRPO; P- and hyperimmune HRPO, respectively) by a modification of the method of Nakane and Kawaoi (17; personal communication). Briefly, 5 mg of HRPO (Sigma HRPO, type VI, RZ 2.75) was dissolved in 1 ml of 0.3 M sodium bicarbonate; 25 μl of 0.32% *p*-formaldehyde was added and stirred for 30 min at room temperature. A 1-ml volume of 0.04 M NaIO₄ was added and stirred continually for 30 min at room temperature. A volume of 1 ml of 0.16 M ethylene

glycol was added, and stirring continued for 1 h at room temperature. The solution was then dialyzed against 1 liter of 0.01 M sodium bicarbonate buffer (pH 9.5) at 4°C overnight. The next day, 10 mg of IgG in approximately 1 ml of 0.01 M bicarbonate buffer (pH 9.5) was added to the solution, and the mixture was stirred for 2 h at room temperature. After the solution was precooled to 4°C in an ice bath, 5 mg of NaBH₄ was added, and the solution was left at 4°C for 2 h. The solution was then dialyzed at 4°C against 1-liter changes of phosphate-buffered saline (PBS) (pH 7.4) for approximately 5 days. The sample was applied to a column (85 by 1.5 cm) of Sephadex G-100 to separate conjugate from free HRPO. Fractions from the first peak were pooled after measurement of the optical density (OD) at 280 nm and 403 nm and calculation of the RZ value ($\text{OD}_{403}/\text{OD}_{280}$). IgG from serum P was also coupled to HRPO exactly as described by Avrameas and Ternynck (1) and separated in a G-100 column. The purified IgG was radiolabeled with ^{125}I or ^{131}I for SPRIA as described previously (19).

Assays. The ELISA test was performed according to the same principles as SPIRA (20). Wells of polyvinyl microtiter plates (Cooke Engineering, Alexandria, Va.) were precoated with 75 μl of a dilution in PBS of a high-titered anti-HA serum and incubated for 4 h at 4°C in a sealed humidified plastic box. After washing (three times, for 5 min each, with PBS containing 0.5% Tween 20 [PBS-T]), the wells were filled with PBS containing 1% bovine serum albumin and left overnight at 4°C . After additional washing, 25 μl of the sample to be tested for HA Ag was added, and the plates were incubated for 20 to 24 h at 4°C . After another washing, 25 μl of PBS-T or diluted serum (to control for specificity or to be tested for anti-HA) was added, and the plates were left for 15 min at room temperature. Without emptying the wells, 25 μl of the conjugate diluted in 50% bovine serum in PBS was added, and the plates were incubated at room temperature for 2 h. After washing, 100 μl of freshly prepared substrate (*ortho*-phenylenediamine [0.4 mg/ml] with 0.006% peroxide in citrate buffer [pH 5.0], made by mixing 98.6 parts of 0.1 M citric acid and 101.4 parts of 0.2 M Na₂HPO₄) was added, and the plates were incubated in the dark at room temperature. After 30 min, 50 μl of 2 M sulfuric acid was added to each well to stop the reaction, and the OD at 493 nm was measured in a double-beam spectrophotometer (Beckman Acta III) equipped with microcuvettes. Unreacted substrate was used as a reference. For specificity testing for HA Ag, six wells (duplicate wells containing PBS-T, presera, or hyperimmune sera) were prepared for each sample. Wells with diluent instead of antigen sample were prepared for both unblocked (PBS-T) and control blocked wells (presera and hyperimmune serum) in each plate. Because the ODs of the wells containing only diluent were higher when blocked with either presera or hyperimmune serum, the following ratio was used to describe the results: $\text{ratio} = (\text{OD}_{\text{test sample}} - \text{OD}_{\text{blank actual}}) / \text{OD}_{\text{blank PBS-T}}$, where OD_{blank actual} represents the OD in wells containing diluent instead of test sample and blocked with either PBS-T, presera, or hyperimmune sera.

When the ratio was calculated for the wells blocked with PBS-T, $OD_{\text{blank actual}} / OD_{\text{blank PBS-T}}$, and the ratio was equal to the commonly used $P/N - 1$. With this ratio, a true negative test sample would give a value of 0, and a value greater than 1.1 was considered positive but specific for HA Ag only if the ratio of results obtained with the hyperimmune serum was less than 50% of the result with the pre-serum.

For detecting anti-HA in sera, a stool with no or only slight nonspecificity as judged by the blocking capacity of pre-sera was used, and 25 μ l of 10-fold dilutions of the serum to be tested was substituted for pre- and hyperimmune sera. The actual titer was calculated by interpolation to 50% of the activity recorded in the wells blocked with pre-serum.

Other serological tests. SPRIA was performed as previously described (20). The blocking test was performed by mixing the serum to be tested with the 125 I-labeled IgG before adding it to the wells. IAHA and IEM were performed as previously described (9, 16).

RESULTS

Conjugation. IgG from a human convalescent serum (P) was labeled with HRPO as described in Materials and Methods, and the conjugate was used in all subsequent experiments. The same IgG was labeled at another time, using 5 mg of IgG instead of 10 mg. Both conjugates had RZ values of 0.48, and both worked equally well in the ELISA for detection of HA Ag in stools. IgG from the hyperimmune chimpanzee serum was also labeled as described in Materials and Methods; this conjugate had an RZ value of 0.49 and gave lower values for negative controls and therefore higher ratios; its ability to detect low HA Ag levels was slightly better. IgG from patient P was also labeled by the two-step glutaraldehyde technique (1), but the sensitivity of tests with this conjugate, which had an RZ value of 0.23, was considerably less.

A blocking SPRIA was performed using a 2% stool filtrate, free of nonspecificity, as the antigen and 10-fold dilutions (to 10^{-6}) of the sera, corresponding IgG preparations, and HRPO conjugates themselves as blocking reagents. The blocking titers, calculated as mentioned in Materials and Methods, are shown in Table 1. The titer, corrected for concentrations, did not change as the IgG's were purified but decreased considerably when the IgG's were labeled to the HRPO. This could be due either to denaturation of the IgG or to the formation of aggregates (3).

Optimal conditions for ELISA. (i) **Precoating.** Three different sera (P, 60, and 67) were tested at a 10^{-2} dilution for precoating the wells. For the P serum and purified IgG the following diluents were tested: PBS, NaCl with 0.1% sodium azide, and 0.05 M carbonate buffer (pH 9.5). The nature of the diluent did not make a difference, and the IgG was no better than

TABLE 1. Anti-HA titer by blocking SPRIA and IAHA during steps of IgG purification and HRPO or fluorescein labeling

Reagents	Anti-HA titer ^a	
	Blocking SPRIA	IAHA
P 14/2 serum	1:7,200	1:128,000
P 14/2 IgG	1:8,500	1:64,000
P 14/2 HRPO conjugate no. 1	1:250	<1:10
P 14/2 HRPO conjugate no. 2	1:260	<1:10
Chimp 753 hyperimmune serum	1:2,700	1:128,000
Chimp 753 hyperimmune IgG	1:5,000	1:16,000
Chimp 753 hyperimmune HRPO conjugate	1:290	<1:10
P 27/4 serum	1:11,000	1:32,000
P 27/4 IgG	1:11,000	NT
P 27/4 FITC ^b conjugate	1:8,000	NT

^a Titer of IgG and conjugate calculated to concentration of 10 mg of IgG per ml. NT, Not tested.

^b FITC, Fluorescein isothiocyanate.

serum. Serum 60, taken from a child 3 months after hepatitis A virus infection, was slightly superior as precoat. Serial 10-fold dilutions of this serum were tested to find the optimal precoat dilution. Dilutions from 10^{-2} to 10^{-4} gave essentially the same results, but the sensitivity for detecting HA Ag diminished considerably at 10^{-5} and was near zero at 10^{-6} . The OD value for the negative control at 10^{-4} was slightly lower when compared to 10^{-2} and 10^{-3} dilutions, and 10^{-4} or $10^{-3.5}$ dilutions of serum 60 in PBS were subsequently used as precoating. Diluting the precoating serum did not help reduce false positive results.

(ii) **Postcoating.** Even after washing with PBS-T, postcoating with 1% bovine serum albumin further lowered the OD for the blank, thereby making the test more sensitive.

(iii) **Incubation time.** The respective precoating and postcoating conditions (4 h at 4°C and overnight at 4°C) were used for all tests in this study. Different times and temperatures were examined for the incubation of the precoated plate with the specimen to be tested for HA Ag. A 24-h incubation at 4°C was clearly superior to 1 or 2 h at room temperature with the precoating conditions used.

Incubation with the HRPO conjugate for 2 h at room temperature proved to be superior to 1 h at 45°C (floating on a water bath), 2 h at 37°C (incubator) or 4°C, 5 h at room temperature or 37°C, and 7 h at room temperature, because of the lower OD value obtained for the negative control. At 4°C the positive value also decreased, resulting in diminished sensitivity.

Twenty-four hours at 4°C proved to be slightly superior but was not routinely used because of inconvenience.

(iv) Conjugate dilution. The conjugate was always diluted in 50% calf serum in PBS, and the optimal final dilution proved to be 1/400 (2.5 µg of IgG per ml).

Substrate. *Ortho*-phenylenediamine proved to be superior to 5-aminosalicylic acid because of the low spontaneous color change and because sulfuric acid could completely stop the reaction, permitting the color to be read for several hours after the addition of the acid. A concentration of 0.4 mg of *ortho*-phenylenediamine per ml proved to be superior to 1 mg/ml. For diluent, 0.15 M citrate buffer (pH 5.0) was superior to PBS, 0.15 M citrate buffer (pH 4.3), or 0.015 M citrate buffer (pH 5.0 or 4.3). A final concentration of peroxide of 0.006% was superior to 0.015%, and there was no advantage in using urea peroxide (0.2 mg/ml) compared to 0.006% peroxide. The incubation time for the substrate proved to be optimal at 30 min as compared with 15 and 45 min.

Specificity. A major problem in detection of HA Ag in stools is specificity. The lack of availability of a good nonprimate hyperimmune anti-HA necessitates the use of human or chimpanzee convalescent (or "hyperimmunized" convalescent) anti-HA sera to detect HA Ag. These sera often have antibody to other stool components and therefore give false positive results. In IEM, specificity is achieved by the morphological identification of the characteristic 27-nm particles with antibody.

In SPRIA we attempted to overcome this problem by performing parallel testing with ¹²⁵I-labeled IgG from paired pre- and hyperimmune

sera and later by dual labeling with ¹³¹I-labeled preexposure IgG and ¹²⁵I-labeled hyperimmune IgG tested simultaneously in the same well. However, since the specific counts due to HA Ag were in many cases much lower than the non-specific counts, it was difficult to interpret the results.

In ELISA another approach was taken. Specificity of the reaction was determined by comparing the blocking effect of the pre- and hyperimmune sera on the binding of HRPO-labeled anti-HA to HA Ag. The unblocked wells were positive if the precoat and the conjugate contained antibody activity to any antigen in the stool, a finding in approximately half of the HA Ag-negative stools tested. After blocking with the preserum, the binding activity remaining should have been due to the HA Ag present in the stool. This could be confirmed by adding the hyperimmune serum to the third pair of wells, thereby blocking all the remaining activity specific for HA Ag. Thus, after blocking with the hyperimmune serum, HRPO activity equaled background levels, and the difference between the activities after blocking with the pre- and hyperimmune sera was the specific activity due to the HA Ag.

Serial stool samples from a patient contracting hepatitis A virus infection after experimental inoculation with the MS-1 strain of virus (4) were tested by ELISA and IEM. As seen in Fig. 1, the unblocked ELISA gave unpredictable results, but the activity remaining in the wells blocked with preserum was found in the same stool samples that contained HA Ag particles as seen by IEM. The hyperimmune serum blocked the activity completely. SPRIA using ¹²⁵I-labeled hyperimmune serum as the conju-

FIG. 1. Serial 2% stool filtrates from a human experimentally infected with the MS-1 strain of hepatitis A virus. ELISA symbols: (Δ) ratio in wells blocked with PBS-T; (□) ratio in wells blocked with preserum; and (○) ratio in wells blocked with hyperimmune anti-HA serum. IEM symbols: Bars represent number of HA Ag particles visualized in 5 squares of a 400-mesh grid.

gate was carried out as well, and the results were similar to the results for the unblocked ELISA.

Table 2 compares the SPRIA, ELISA, and IEM results from different stools collected from patients who contracted hepatitis A virus infection during the San Diego outbreak (7). Serial stool samples collected during the acute phase of illness were selected from nine patients in whom HA Ag particles had been detected by IEM in at least one stool filtrate. In addition, stools from all eight patients from whom stools were collected 3 months after the acute illness were studied as negative controls. The same filtrates were tested by all three techniques.

Of 37 acute-phase stools tested, 16 contained specific HA Ag activity by ELISA; 13 of these contained HA Ag particles as judged by IEM, but the remaining 3 stools did not. Of the 17 in which HA Ag was visualized by IEM, 13 were specifically positive for HA Ag by ELISA, and 1 was not available for testing. The remaining three stools (Hd 10/14, Mo 10/20, and Wo early) contained activity in the unblocked wells that was only partially blocked by the presera. However, since the hyperimmune serum failed to diminish the ratio to below 50%, the remaining activity was interpreted as not being specific for HA Ag. None of the eight control stools contained specific HA Ag activity.

Sensitivity. It was found previously (unpublished data) that daily stools collected from certain chimpanzees experimentally infected with hepatitis A virus contained little or no nonspecificity, and it was therefore possible to detect HA Ag in the stools by SPRIA. To compare the sensitivity of SPRIA, ELISA, and IEM, daily stools from chimpanzee 753 were tested by each method.

As seen in Table 3, ELISA and SPRIA were approximately equally sensitive in detecting HA Ag in the 2% filtrates when the same reagents were used. ELISA performed with serum P as precoat and hyperimmune HRPO as conjugate was positive from day 13 to day 21 after inoculation and was also positive on day 25. SPRIA performed with the same reagents was positive from day 13 to day 21 but not on day 25. ELISA was also performed using serum 60 as precoat and P-HRPO as conjugate. In this test, positive values for HA Ag were obtained from day 13 to day 20 (data not shown). The IEM results on the same stool filtrates are also tabulated, and it can be seen that ELISA and SPRIA were positive 2 days before HA Ag particles could be seen by IEM, whereas HA Ag could be detected by IEM 2 to 3 days after the ELISA and SPRIA became negative.

The sensitivity of ELISA was also compared

to that of SPRIA by testing dilutions of purified HA Ag and a strongly positive stool which contained no nonspecific activity (Hs 10/12). As seen in Table 4, ELISA (using serum 60 as precoat and P-HRPO as conjugate) was positive at two- to fourfold higher dilutions but, if the specificity testing for HA Ag in ELISA is taken into account, the two tests are of equal sensitivity.

Blocking ELISA. Using a stool filtrate with essentially no nonspecific activity, anti-HA in paired sera was measured by using dilutions of these sera as blocking agents. In Table 5, ELISA anti-HA titers, calculated as described in Materials and Methods, are compared with IAHA titers and IEM measurements of the same sera. All presera from the humans and chimpanzees contracting hepatitis type A lacked blocking activity in ELISA, whereas the acute- and convalescent-phase sera were positive for antibody. In the three human serum pairs tested, there were titer rises from the acute to convalescent specimen of only two- to threefold. In the chimpanzees there were no titer rises. Pre- and convalescent sera from two patients contracting hepatitis type B or non-A, non-B who had no anti-HA by IAHA did not block in ELISA.

DISCUSSION

Tests for detecting HA Ag and anti-HA by using IEM and HA Ag-containing stool filtrates have been available since 1973. Subsequently, complement fixation tests and IAHA were developed for detection of anti-HA by using HA Ag derived from livers of marmosets acutely ill with hepatitis type A (15, 18). Because these animals are not generally available for experimental use, Moritsugu et al. purified HA Ag from human stool for use in IAHA tests (16). This required a fairly substantial purification procedure, with initial monitoring of the stools by IEM. The complexity of the purification procedures plus the general shortage of HA Ag-containing stools have prevented many investigators from studying type A hepatitis. Many of these difficulties can be overcome by the ELISA and blocking ELISA tests described here because HA Ag can be detected specifically in crude stool suspensions and because HA Ag-containing stools without nonspecific reaction can be used as an antigen source in blocking ELISA for anti-HA detection in sera.

When paired pre- and hyperimmune sera are used in control blocking experiments for specific detection of HA Ag, one assumes that the only difference between the two sera is the anti-HA activity and that the two sera in other respects are identical. Although this assumption was not

TABLE 2. *Specific detection of HA Ag in 2% stool filtrates by ELISA, SPRIA, and IEM*

2% stool filtrates ^c	ELISA ^a			Result	SPRIA ^b		Result	IEM (HA Ag particles/ 5 squares)
	Ratio				P/N			
	PBS-T	PRE	HYPER		PRE	HYPER		
Acute								
Hs 10/12	48.7	63.1	<0	+	1.1	69.6	+	512
10/13	65.1	59.0	1.2	+	2.7	35.6	+	450
10/14	62.1	61.9	0.3	+	1.5	66.7	+	433
10/15	46.4	41.1	<0	+	1.1	18.5	+	201
10/18	7.9	0.9	<0	0	1.5	2.1	0	0
10/19	110.7	5.5	10.2	0	7.2	8.6	0	0
10/20	66.4	3.4	7.4	0	4.3	4.9	0	0
Su 10/11	56.0	57.1	<0	+	1.2	14.7	+	636
10/13	0.4	0.0	<0	0	0.8	1.6	0	0
10/17	5.0	0.0	<0	0	1.6	2.2	0	0
Hd 10/11	37.5	33.2	6.0	+	1.8	8.9	+	100
10/13	121.1	112.3	11.6	+	3.3	81.2	+	246
10/14	6.2	4.6	2.4	0	0.3	5.5	+	6
AS 10/8	159.3	60.9	10.0	+	10.8	31.9	+	85
10/10	123.3	27.8	6.4	+	6.1	13.2	+	639
10/11	30.0	21.6	<0	+	2.1	10.2	+	40
10/14	4.3	0.9	<0	0	0.9	1.5	0	0
10/15	0.5	0.5	<0	0	1.7	1.6	0	0
10/20	0.7	0.5	<0	0	1.3	1.4	0	0
Ag 10/13	49.8	14.2	3.6	+	3.8	6.3	0	8
10/15	144.0	14.7	18.2	0	8.6	11.6	0	0
10/16	7.5	2.9	<0	+	1.8	2.1	0	0
MG 10/10	3.4	2.7	<0	+	1.0	3.0	+	16
10/11	0.2	0.2	<0	0	0.9	1.2	0	0
10/12	7.3	5.5	<0	+	1.5	5.0	+	0
10/15	5.9	0.2	<0	0	1.7	2.2	0	0
10/18	0.0	<0	<0	0	0.7	0.8	0	NT
Mo 10/18	88.0	43.8	11.8	+	9.2	27.4	+	150
10/20	42.2	17.3	10.4	0	2.2	5.6	+	87
Wo early	125.7	10.3	20.1	0	17.3	22.1	0	3
10/13	1.3	<0	<0	0	0.6	1.3	0	0
10/13	0.3	<0	<0	0	0.8	0.8	0	0
10/14	0.1	<0	<0	0	1.2	1.5	0	0
Kj 10/12	NT	NT	NT	NT	NT	NT	NT	11
10/13	5.4	0.3	<0	0	2.1	2.4	0	0
10/14	1.0	<0	<0	0	1.2	1.4	0	0
10/14	12.0	1.7	0.1	+	1.2	1.9	0	0
10/20	3.1	<0	<0	0	2.5	2.7	0	0
Convalescent ^d (3 months)								
Ma Jan	136.5	8.2	16.2	0	NT	NT	NT	0
La Jan	18.7	0.4	0.1	0	NT	NT	NT	0
Va Jan	0.6	<0	<0	0	NT	NT	NT	0
Re Jan	10.1	0.3	<0	0	NT	NT	NT	0
Wa Jan	0.1	<0	<0	0	NT	NT	NT	0
Sm Jan	445.8	21.2	50.2	0	NT	NT	NT	0
Gr Jan	97.9	11.5	12.6	0	NT	NT	NT	0
Jo Jan	1.2	<0	<0	0	NT	NT	NT	0

^a ELISA (serum 60 as precoat and P-HRPO as conjugate) control blocked with PBS-T; preserum (PRE) and hyperimmune serum (HYPER) from chimpanzee 753. Results were considered specifically positive for HA Ag (+) only if the preserum blocked ratio was ≥ 1.1 and the hyperimmune serum blocked ratio was 50% lower. NT, Not tested.

^b SPRIA with mixture of ^{131}I -labeled IgG from preserum (PRE) and ^{125}I -labeled IgG from hyperimmune serum (HYPER) from chimpanzee 753. Results were considered specifically positive (+) for HA Ag if P/N ≥ 2.1 with HYPER and P/N with PRE was 50% lower.

^c Serial stools were collected during the San Diego outbreak of hepatitis type A from nine patients. At least one stool from each patient was found positive for HA Ag by IEM.

^d Stools from eight patients were collected 3 months after the San Diego outbreak and were studied as negative controls.

TABLE 3. Comparison of sensitivity of ELISA, SPRIA, and IEM for detecting HA Ag

2% stool filtrates (day) ^c	ELISA ^a			SPRIA ^b			IEM (HA Ag particles/ 5 squares)	
	Ratio			Result	P/N			Result
	PBS-T	PRE	HYPER		PRE	HYPER		
-15	0.5	0.2	<0	0	1.0	1.1	0	0
5	0.2	0.3	<0	0	0.8	1.1	0	NT ^d
11	0.6	0.3	<0	0	1.2	1.3	0	0
12	0.0	0.0	<0	0	0.6	1.4	0	0
13	1.6	1.4	<0	+	0.9	3.1	+	0
14	2.1	2.0	<0	+	1.1	3.9	+	0
15	1.7	2.0	<0	+	0.9	3.1	+	19
16	5.1	5.6	0.5	+	1.1	8.3	+	56
17	10.4	9.9	2.0	+	1.1	16.8	+	86
18	9.1	9.2	2.3	+	0.9	7.7	+	34
19	NT	NT	NT		1.1	8.4	+	26
20	5.6	8.7	0.9	+	1.2	6.2	+	29
21	2.7	3.5	1.5	+	1.2	3.6	+	20
22	0.5	0.5	<0	0	1.3	2.3	0	25
23	0.8	0.8	<0	0	1.1	1.3	0	23
24	0.7	0.9	<0	0	1.0	1.4	0	7
25	1.4	1.6	0.0	+	1.2	1.9	0	3
26	0.5	1.0	<0	0	1.1	1.7	0	0
27	0.2	0.6	0.4	0	0.7	1.4	0	0
28	0.4	0.4	<0	0	1.5	1.3	0	0
29	<0	0.1	<0	0	1.1	1.1	0	0
30	0.1	0.1	<0	0	1.0	1.2	0	0
31	0.2	0.3	<0	0	1.2	1.5	0	NT
40	0.3	0.5	<0	0	1.2	1.0	0	NT

^a ELISA with serum S as precoat and chimpanzee 753 hyperimmune HRPO-labeled IgG as conjugate; blocked with PBS-T, preserum (PRE), and hyperimmune serum (HYPER) from chimpanzee 753. Results were considered specifically positive for HA Ag (+) only if the preserum blocked ratio was ≥ 1.1 and the hyperimmune serum blocked ratio was $\geq 50\%$ lower.

^b SPRIA with mixture of ^{131}I -labeled IgG of preserum (PRE) and ^{125}I -labeled IgG of hyperimmune serum (HYPER) from chimpanzee 753. Results were considered specifically positive for HA Ag only if P/N ≥ 2.1 with HYPER and P/N with PRE was 50% lower.

^c 2% stool filtrates collected from chimpanzee 753 during experimental infection with hepatitis A. Chimpanzee was inoculated on day 0, serum glutamic pyruvic transaminase rise occurred on day 22 to 33, and anti-HA was first detected by IEM on day 23.

^d NT, Not tested.

absolutely proven, the paired sera approach gave results consistent with this hypothesis when compared with the IEM results.

From the preliminary results presented it is difficult to judge exactly the specificity of the test, but, of eight negative control stool samples, none was specifically positive for HA Ag by ELISA. Of the serial samples taken from patients with acute hepatitis type A, 3 of 16 samples negative by IEM were positive by ELISA. However, since the sensitivity of ELISA is comparable to IEM, and different factors affect the sensitivity of the two tests, these cannot be judged as false positive. There were 3 of 17 samples positive by IEM that were not specifically positive by ELISA. However, all three of those stools contained nonspecific activity that may have masked the HA Ag in the samples. Of the eight negative control samples, three were

strongly positive in the PBS-T blocked wells, and neither the preserum nor the hyperimmune serum was able to block it completely. In the two cases with the strongest activity, the hyperimmune serum did not block as well as the preserum, suggesting that the antibody titer against some of the nonspecific activity may have diminished by the time the hyperimmune serum was collected. This situation may explain the failure of ELISA to detect the three IEM-positive samples (Table 3, Hd 10/14, Mo 10/20, and Wo early). Paired sera separated by as short a time interval as possible might minimize this problem.

Recently Hall et al. have reported the detection of HA Ag in 5% human stool extracts by SPRIA and found it sensitive but not specific for HA Ag; 5 of 16 samples from asymptomatic individuals were positive by SPRIA but negative

by IEM (10). This discrepancy could be due to a higher sensitivity of SPRIA than IEM. However, no testing for specificity was reported. Hall et al. reported only 8% false positive results in several hundred primate and human fecal extracts. We find that the nonspecific activity which is common in human stools is rare in primate stools.

The amount of nonspecificity is of course dependent upon the antibody distribution in the precoating serum and the conjugate used as well as upon the variety of antigens present in the stools, but we have found blocking antibody against the stool-associated nonspecificity in most sera tested. Therefore, specificity controls must be included in these types of assays.

Both the ELISA and the SPRIA depend on the ability of the HA Ag in the stool to bind anti-HA. It is possible, however, to detect HA Ag particles by IEM even if they are already covered with antibody. This might explain why IEM detected HA Ag particles for 2 to 3 days after SPRIA and ELISA became negative, even though SPRIA and ELISA tests were able to detect HA Ag in the stools before IEM.

The anti-HA titers by blocking ELISA presented in Table 5 show the specificity of the test when stool filtrates were used as antigen. The

titers are equivalent to results obtained with blocking SPRIA using purified HA Ag as antigen (20) and in both cases (as well as by IEM) early antibody, possibly IgM, was detected well before anti-HA was detected by IAHA.

Of practical interest are the results obtained with paired acute and convalescent sera. A rise in anti-HA titer is the best available way to confirm the diagnosis of hepatitis type A cases. All three human cases of hepatitis A virus infection had only a two- to threefold rise from acute to convalescent sera because high titers of anti-HA were already present during the acute phase of illness (Table 5). Further studies have to be done to determine whether this is a consistent finding. However, presera uniformly had no detectable anti-HA.

It has previously been proposed that the diagnosis of hepatitis type A could be made on a single acute-phase serum if IEM were positive for anti-HA and IAHA were negative (21); IAHA usually could not detect anti-HA earlier than 2 to 6 weeks after the acute phase, whereas IEM could detect anti-HA in the acute phase of illness. Blocking ELISA or SPRIA can be substituted for IEM for detection of early antibody; in all four hepatitis type A cases where acute sera were tested, a tentative diagnosis could be made

TABLE 4. Comparisons of sensitivity of ELISA with SPRIA for detection of HA Ag

Dilutions	ELISA ^a ratio			SPRIA P/N	
	PBS-T	PRE	HYPER	ELISA reagents ^b	Usual reagents ^c
2% stool filtrate ^d					
1:1	38.1	36.6	<0	13.0	10.2
1:2	36.0	34.4	<0	16.3	15.3
1:4	27.4	26.2	<0	11.1	11.8
1:8	15.1	13.6	0.4	4.8	6.0
1:16	6.3	5.8	<0	3.1	4.3
1:32	2.3	3.0	<0	1.8	2.8
1:64	1.1	0.3	0.2	1.3	1.7
Purified HA Ag					
1:50	6.2	3.5	0.1	3.1	3.9
1:100	3.3	2.1	0.4	3.1	3.2
1:200	2.5	0.9	<0	1.5	1.8
1:400	1.6	1.0	0.8	1.4	1.4
1:800	0.4	0.4	<0	1.2	1.5
1:1,600	<0	0.4	0.8	1.1	1.1
1:3,200	0.6	0.5	1.2	0.9	1.2

^a ELISA with serum 60 as precoat and P-HRPO conjugate blocked with PBS-T; preserum (PRE) and hyperimmune serum (HYPER) from chimpanzee 753. Results were considered specifically positive for HA Ag only if the preserum blocked ratio was ≥ 1.1 and the hyperimmune serum blocked ratio was 50% lower.

^b SPRIA with same serum and IgG as ELISA (serum 60 as precoat and P IgG labeled with ¹²⁵I as conjugate). P/N ≥ 2.1 is considered positive.

^c SPRIA with usual serum and IgG combination (serum S as precoat and chimpanzee 753 hyperimmune IgG labeled with ¹²⁵I as conjugate). P/N ≥ 2.1 is considered positive.

^d 2% human stool filtrate with no detectable nonspecificity; contained 512 hepatitis A particles per 5 squares by IEM.

TABLE 5. Comparison of blocking ELISA, IAHA, and IEM for detection of anti-HA

Sera from cases of:	Anti-HA by:		
	Blocking ELISA ^a	IAHA ^b	IEM ^c
Type A hepatitis			
B day 0	<1:10	<1:10	0
B day 40 (acute)	1:1,300	<1:10 ^d	2+
B day 105	1:3,200	1:1,600	NT
K day 0	<1:10	<1:10	0
K day 37 (acute)	1:1,100	<1:10	1+
K day 100	1:2,700	1:5,000	3+
We acute	1:4,900	<1:10	1+
We 3 months	1:120,000	1:16,000	3-4+
Chimp 753 day 4	<1:10	<1:10	0
day 33 (acute)	1:2,100	<1:10	2-3+
day 153	1:1,700	1:3,200	3-4+
Chimp 714 day 5	<1:10	<1:2	NT
day 153	1:3,000	1:3,200	NT
Type B hepatitis			
Preexposure	<1:10	<1:10	0
Convalescent (7 months)	<1:10	<1:10	0
Type non-A, non-B hepatitis			
Acute	<1:10	<1:40 ^d	NT
Convalescent (2 months)	<1:10	<1:100 ^d	NT

^a Blocking ELISA titers calculated as described in Materials and Methods.^b Titers.^c Antibody rating on a scale of 0 to 4+. 0, No antibody; 4+, heavily coated with antibody; NT, not tested.^d Nonspecific reaction at this dilution.

on the difference in anti-HA activity between blocking ELISA or SPRIA and IAHA, as well as on the difference between IEM and IAHA.

Since the first report by Engval and Perlmann (8) and Van Weemen and Schuurs (23), ELISA has been increasingly used as an alternative to RIA. The advantages of ELISA over RIA are the lack of radiation safety hazard, the stability of the conjugate, and the ability to detect the final result by eye or by a relatively inexpensive spectrophotometer instead of by expensive radiation counting equipment.

Assays employing enzyme-coupled antibody have now been developed in many fields, including virology, tropical diseases, veterinary medicine, endocrinology, and pharmacology, but there have been only a few reports which compared the ELISA with its closest competitor, RIA (24).

Wolters et al. developed an ELISA for detecting hepatitis B surface antigen and, though using different antisera, found the same sensitivity as the Ausria RIA test (25). Yolken et al. have reported approximately equal sensitivity and

specificity of SPRIA and ELISA for detection of the human reovirus-like agent of infantile gastroenteritis by using the same serum for conjugation in the two tests (26). Their ELISA results also agreed 100% with the findings of reovirus-like particles by IEM.

Voller et al. compared SPRIA and ELISA for detection of antibody to different parasitic antigens, using the same anti-human IgG for conjugation, and found equal sensitivity and specificity (23a). Others have found ELISA to be less sensitive than the comparable RIA (24). Our results confirm that equal sensitivity and specificity can be obtained when the same source of reagents is used.

One of the main problems in developing an ELISA is to couple the enzyme to the antibody without destroying the latter's immunological activity and, at the same time, to achieve a reasonable degree of conjugation. Our initial efforts in labeling HRPO to anti-HA IgG completely destroyed the anti-HA activity. Subsequently, by following the method of Nakane and Kawaoi with extreme care, we were able to make

conjugates useful in ELISA. However, the specific anti-HA activity as judged by blocking SPRIA was considerably decreased. This partial destruction of immunological activity is in contrast to the original report of Nakane and Kawaoi (17) but corresponds well with the finding of Boorsma et al. (3). In the same blocking test, the titer of corresponding serum, IgG, and fluorescein isothiocyanate-labeled IgG was measured, and little or no loss of antibody activity was detected after fluorescein isothiocyanate labeling (13). Further work must be done to optimize the conjugation procedure, especially when relatively low-titered convalescent serum is used as starting material.

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