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Solid-Phase Enzyme-Linked Immunosorbent Assay for Detection of Hepatitis A Virus

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An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of hepatitis A virus in human fecal specimens. Investigations with 88 fecal specimens from 77 patients with suspected viral hepatitis and 8 of their household contacts showed that ELISA was as specific and sensitive as radioimmunoassay and almost as sensitive as immune electron microscopy. The ELISA is quick and simple to perform, does not require sophisticated technical equipment, and can be read with the naked eye, making it suitable for field work and rapid diagnosis.

The lack of a serological marker of infection has until recently impeded progress in the understanding of hepatitis A. In 1973, Feinstone et al. (3), using the technique of immune electron microscopy (IEM), detected 27-nm virus-like particles in acute-phase fecal specimens of patients with hepatitis A; this particle is now officially designated the hepatitis A virus (HAV), and antibody to it is designated as anti-HAV (16).

The use of IEM for detecting HAV has been a limiting factor in the study of the disease, since the technique is difficult to perform and requires highly trained personnel and access to an electron microscope. Two other tests, immune adherence hemagglutination (9, 11) and solid-phase radioimmunoassay (SPRIA) (4, 14), have been developed for detection of HAV which are similar in sensitivity to IEM and more applicable to mass screening of specimens. Both methods, however, require the use of partially purified specimens, and SPRIA involves the use of radioactive reagents and radiation counting equipment, thus making the techniques impractical in many situations.

The enzyme-linked immunosorbent assay (ELISA), originally developed by Engvall and Perlmann (2), has been successfully used for detection of hepatitis B surface antigen in human serum (15), herpes simplex virus from clinical material (10), and the human reovirus-like agent of infantile gastroenteritis in feces (17). This paper reports the use of the ELISA technique for detection of HAV.

MATERIALS AND METHODS

Patients and specimens. Fecal specimens were obtained from 77 patients with suspected acute viral hepatitis, admitted to Fairfield Hospital during the period February 1975 to March 1976, and from 8 household contacts. Paired acute- and convalescent-phase sera were also collected from each subject. All specimens were stored at −20°C until they could be tested.

Diagnostic criteria. A diagnosis of viral hepatitis was made by specialist physicians on the basis of clinical assessment aided by liver function tests. A patient was regarded as having hepatitis A if HAV was detected in the feces during the acute phase of the illness, and/or a rising anti-HAV titer was demonstrable in the paired sera, and/or hepatitis A-specific immunoglobulin M (IgM) was detected in acute-phase sera (6, 8). Tests for HAV and anti-HAV were performed by SPRIA as previously described (5). A diagnosis of hepatitis B infection was made if a transitory hepatitis B surface antigenemia or a primary anti-hepatitis B surface antigen response was detected by SPRIA (Abbott Laboratories, North Chicago, Ill.). No tests for antibody to the hepatitis B core antigen or the hepatitis B-specific DNA polymerase were performed.

Reagents. Preinfection and postinfection sera were available from one patient (AC) with laboratory-acquired, serologically confirmed hepatitis A. Late convalescent-phase sera were also collected, 4 months after onset of dark urine, from two other patients (TG and SF) with documented hepatitis A infection. IgG was purified from 1.0-ml samples of serum TG by precipitation with ammonium sulfate followed by chromatography on diethylaminoethyl-cellulose, as described by Purcell et al. (13). Purified IgG was stored at −20°C.

Test methods. Fecal extracts were prepared as 20% (wt/vol) suspensions in phosphate-buffered saline (PBS; pH 7.4) and clarified by centrifugation at 16,000 × *g* for 60 min at 4°C. Supernatant fluids were then concentrated by ultracentrifugation at 100,000 × *g* for 3 h at 4°C in a Beckman model L5-65 preparative ultracentrifuge, using a type 45 Ti rotor. The resultant pellets ("Spinco pellets") were resuspended in PBS to approximately one-tenth their original volume. Prior to testing each fecal specimen for HAV by IEM,

SPRIA, and ELISA, the Spinco-pelleted fecal extracts were diluted 1:4 in PBS and extracted with an equal volume of chloroform (British Drug Houses). The aqueous layer was carefully removed and stored at −20°C until tested.

Two fecal specimens (specimens 1 and 2, Table 1) containing HAV were partially purified by isopycnic ultracentrifugation in cesium chloride (CsCl). A 0.5-ml volume of each chloroform-extracted fecal specimen was layered on top of 4.0-ml 20 to 50% (wt/vol, in 0.05 M phosphate buffer, pH 7.4) linear CsCl gradients and centrifuged at 275,000 × *g* for 24 h at 4°C, using an SW 60 rotor in a Beckman model L5-65 preparative ultracentrifuge. Twenty fractions were collected from the bottom of each tube, and the density of each was determined from its refractive index, as described previously (7).

IEM test. For detection of HAV by IEM, 1.0-ml volumes of chloroform-treated fecal extracts or 0.1-ml volumes of undiluted CsCl gradient fractions were reacted with 0.1 ml of a 1:20 dilution of human postinfection hepatitis A serum (patient AC) rated 4+ for anti-HAV (8). After incubation at 37°C for 1 h and then overnight at 4°C, the reaction mixtures were centrifuged at 37,000 × *g* for 90 min. Supernatant fluids were carefully removed, and the deposits were negatively stained with 4% phosphotungstic acid, pH 7.4, and examined immediately in a Philips EM 301 electron microscope at a plate magnification of approximately 44,000. All specimens were examined under code, by a single operator (S.A.L.), on EM 400 mesh grids. Fecal extracts containing 27-nm particles complexed by antibody were then re-examined with 0.1 ml of a 1:20 dilution of human preinfection hepatitis A serum (AC). A fecal specimen was regarded as positive for HAV only when it contained 27-nm particles which were complexed by the postinfection, but not by the preinfection, hepatitis A serum. The number of particles visualized in four electron microscope grid-squares were counted, and the average number per grid-square was calculated.

SPRIA test. The SPRIA method used for detection of HAV was a modification of the method described by Purcell et al. (14). For the test, wells of polyvinyl microtiter plates (Cooke Engineering Inc., Alexandria, Va.) were coated with a 1:1,000 dilution of human convalescent-phase hepatitis A serum (patient SF) in 0.85% NaCl (saline). After incubation for 4 h at 20°C, the wells were washed with saline, filled with 1% bovine serum albumin in saline, and allowed to stand at 4°C overnight. The wells were washed three times with PBS and then inoculated in duplicate with 25 μl of the sample to be tested.

In this first part of the SPRIA test, the SPRIA screening test, the plates were incubated at 4°C for 16 h, washed three times with PBS, and then inoculated with 50 μl of ^{125}I-labeled human anti-HAV IgG (patient TG) diluted in bovine serum (14). After washing the wells six times with PBS, plates were cut with a hot spatula, and each well was tested for residual radioactivity. Positive and negative controls, established in a previous study (6, 8), were included for each plate. The residual counts per minute detected in wells containing test samples were divided by the mean residual counts per minute in wells containing negative controls and PBS. The residual counts per minute in wells containing the negative control fecal specimens was comparable to the residual counts per minute in wells containing PBS. The result was expressed as a ratio of test sample counts per minute to negative control counts per minute, and values of 2.1 or greater were regarded as positive (P/N).

The specificity of each positive specimen was then determined by differential neutralization, using pre- and postinfection sera from a chimpanzee experimentally infected with HAV (1). Equal volumes of the positive sample were mixed with a 1:100 dilution of pre- or postinfection chimpanzee serum and incubated at 37°C for 1 h and then overnight at 4°C. Next day, a 25-μl sample was inoculated into anti-HAV-coated plates, and the test was continued as for the direct SPRIA test above. A 40% or more reduction in residual radioactivity by the postinfection serum when compared with the preinfection serum was considered as evidence for specific neutralization.

ELISA assay. The method used was essentially that of Wolters et al. (15). The test used the same batch of antibody-coated polyvinyl microtiter plates and convalescent human anti-HAV IgG (TG) as used in the SPRIA test. Horseradish peroxidase (RZ ≃ 3.0, Sigma type VI) was coupled to convalescent IgG from patient TG by the method of Nakane and Kawaoi (12). The substrate was prepared by dissolving 80 mg of 5-aminosalicylic acid (Merck, Munich) in 100 ml of hot distilled water, the pH was adjusted to 6.0 with 1 N NaOH, and then 9 parts were added, to 1 part of 0.05% (vol/vol) H_2O_2 and used immediately.

In the ELISA screening test, anti-HAV-coated plates were inoculated with 25 μl of the sample under test, incubated at 4°C for 16 h, washed three times with PBS, and inoculated with 50 μl of peroxidase-labeled human anti-HAV IgG (TG) diluted 1:100 in PBS. The plates were incubated for a further 2 h at 37°C and washed three times with PBS, and then 50 μl of substrate was added. The plates were incubated in the dark at room temperature for 30 min, the enzyme-substrate reaction was stopped by the addition of 1 N NaOH, and the amount of brown color in the substrate solution produced by the action of the peroxidase bound to the wells was determined. These readings were performed with the naked eye and scored on a 0 to 3+ scale: 0, no color reaction; 1+, a light to medium color; 2+, a medium to strong color; 3+, a very strong color reaction. A 1+ difference in reading was considered significant. All tests were performed in duplicate, and each plate was coded and read independently by two operators.

The specificity of each positive result was tested by a blocking technique. In this test, the anti-HAV-coated plates were inoculated with 25 μl of the test sample, and the plates were incubated for 16 h and then washed with PBS. Next, 25 μl of a 1:10 dilution of pre- or postinfection human hepatitis A serum (AC) was added to each of two wells. The blocking reaction proceeded overnight at 4°C; the plates were then washed three times with PBS, inoculated with 50 μl of peroxidase-labeled human anti-HAV IgG, and processed as above for the ELISA screening test. Specific blocking was regarded to have occurred if no color developed in the wells containing postinfection serum

while the wells containing preinfection serum were still positive on the 1 to 3+ scale. All tests were performed in duplicate, and the plates were scored under code by two operators.

RESULTS

Examination of fecal specimens. After correlation of the results, the 85 subjects were allocated to four groups: (i) hepatitis A (33 patients); (ii) hepatitis B (19 patients); (iii) viral hepatitis without serological evidence of infection with either HAV or hepatitis B virus (12 patients); (iv) miscellaneous (13 patients with diseases other than viral hepatitis and 8 household contacts). The 88 fecal specimens obtained from these 85 subjects were examined for HAV by IEM, SPRIA, and ELISA.

(i) IEM. Twenty specimens contained 27-nm particles morphologically similar to HAV which were complexed by postinfection but not by preinfection human hepatitis A sera (AC) (see Table 1). The 20 patients from whom these fecal specimens were collected all showed rising levels of anti-HAV between their acute and convalescent sera and had detectable levels of hepatitis A-specific IgM in their acute-phase sera.

Four other specimens contained 27-nm particles of similar morphology to HAV, which were complexed by both the pre- and postinfection human hepatitis A sera. One of these specimens was collected from a patient with serologically confirmed hepatitis A infection; the other three were obtained from patients with liver disease other than viral hepatitis.

(ii) SPRIA. In the SPRIA screening test, 53 specimens were positive, but of these only 18 were specifically neutralized by postinfection chimpanzee serum. Each of these specimens contained HAV detectable by IEM (Fig. 1, Table 1).

Of the 36 samples which were not differentially neutralized, two were positive for HAV by IEM (Fig. 1; specimens 16 and 17, Table 1), and both of these contained fewer than 10 HAV particles per electron microscope grid-square. In these specimens, addition of postinfection chimpanzee serum reduced the radioactivity 10 and 24%, respectively, but not to the 40% cut-off chosen as significant for specific neutralization.

In the remaining 34 positive samples which could not be neutralized and which were negative for HAV by IEM, addition of postinfection chimpanzee serum usually produced some reduction in counts. In 26 specimens this ranged from 0 to 19% and in 8, from 20 to 29%. There was no correlation between percentage of reduction and type of illness (Fig. 1).

Table 1 compares SPRIA and IEM for detection of HAV in fecal specimens.

(iii) ELISA. In the ELISA screening test, 59 specimens were scored positive: of these, 10 specimens had readings of 1+, 14 had readings of 2+, and 35 were scored 3+. Only 17 specimens showed specific blocking with the postinfection human serum, and each contained HAV detectable by IEM (Table 1, Fig. 1). There was no

TABLE 1. *Results of IEM, SPRIA, and ELISA tests on fecal specimens from 20 patients with serologically confirmed hepatitis A*

Specimen	HAV by IEM[a]	SPRIA		ELISA	
		Screen (P/N)	Neutralization[b] (% reduction)	Screen[c]	Blocking[d]
1	+ (50)	19.3	+ (90)	2+	+
2	+ (100)	21.0	+ (88)	3+	+
3	+ (10)	12.2	+ (43)	2+	+
4	+ (5)	2.7	+ (42)	3+	−
5	+ (100)	45.9	+ (58)	3+	+
6	+ (100)	30.2	+ (90)	2+	+
7	+ (100)	14.3	+ (48)	3+	+
8	+ (100)	35.5	+ (49)	3+	+
9	+ (10)	59.1	+ (83)	3+	+
10	+ (50)	19.4	+ (78)	1+	+
11	+ (50)	20.9	+ (77)	1+	+
12	+ (20)	34.3	+ (69)	2+	+
13	+ (10)	45.0	+ (49)	3+	−
14	+ (30)	64.2	+ (83)	3+	+
15	+ (300)	15.2	+ (86)	2+	+
16	+ (5)	68.5	− (10)	1+	−
17	+ (5)	36.9	− (24)	2+	+
18	+ (50)	20.6	+ (82)	3+	+
19	+ (100)	10.7	+ (45)	1+	+
20	+ (20)	3.5	+ (71)	1+	+

[a] (Titer) expressed as the number of HAV particles visualized per electron miroscope grid-square.

[b] Forty percent or greater reduction in radioactivity by the postinfection serum when compared with the preinfection chimpanzee serum was scored as positive neutralization.

[c] Degree of color scored on 0 to 3+ scale as described in Materials and Methods.

[d] Color reduction from positive (1 to 3+ rating) to negative (0 rating) when the specimen was reacted with pre- and postinfection human serum, respectively, was scored as specific blocking.

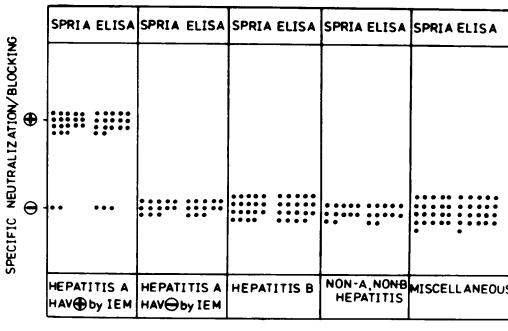

FIG. 1. *Comparison of IEM, SPRIA, and ELISA for detection of HAV in 88 fecal specimens from 85 subjects.*

correlation between the amount of color in the screening test and the probability that a specimen would be specifically blocked. There was minimal correlation between the amount of color developed in the ELISA and the number of particles detected by IEM (Table 1).

HAV was detectable by IEM in 3 of the 42 samples which could not be specifically blocked (Table 1). Each of these samples contained fewer than 10 HAV particles per electron microscope grid-square by IEM. Two specimens, positive for HAV by IEM and SPRIA, were negative by ELISA, and one specimen, positive by IEM and ELISA, was negative for HAV by SPRIA. Table 1 compares ELISA and SPRIA for the detection of HAV in the 20 IEM-positive fecal specimens.

The 68 specimens which were negative for HAV by IEM were not specifically neutralized by SPRIA or blocked by ELISA (Fig. 1).

Comparison of IEM, SPRIA, and ELISA for detection of HAV in fecal specimens. The ELISA blocking test was then compared to the SPRIA neutralization procedure and IEM test for detection of HAV in serial dilutions of a fecal specimen containing HAV (specimen 2, Table 1). IEM detected HAV to a titer of 1:1,000, whereas SPRIA and ELISA both showed specific neutralization and blocking to titers of 1:100 (Fig. 2).

Detection of partially purified HAV in CsCl gradients by IEM, SPRIA, and ELISA. Two fecal specimens (specimens 1 and 2, Table 1) were centrifuged to isopycnic conditions in CsCl as described above. The undiluted gradient fractions were examined, under code, by IEM, SPRIA, and ELISA. Coincident peaks of particles, radioactivity, and coloring with a mean buoyant density of 1.34 g/cm^3 were detected. Similar profiles were obtained by each technique on both specimens. Figure 3 shows the IEM, SPRIA, and ELISA profiles for specimen 1.

Diagnostic value of detection of HAV in Fecal specimens by IEM, SPRIA, and ELISA. Detection of HAV by IEM was found in 61% (20 of 33) of patients with serologically confirmed hepatitis A, whereas SPRIA and ELISA detected HAV in 55% (18 of 33) and 52% (17 of 33), respectively. Fecal specimens were collected from all of the 33 patients within 1 week from the onset of dark urine. All 33 patients showed rising levels of anti-HAV between paired sera as well as the presence of hepatitis A-specific IgM in acute-phase sera as detected by SPRIA.

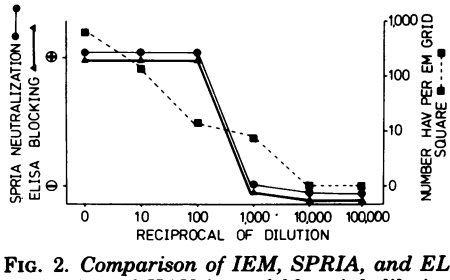

FIG. 2. *Comparison of IEM, SPRIA, and ELISA for detection of HAV in 10-fold serial dilutions of specimen 2 (Table 1).*

DISCUSSION

The above results demonstrate that ELISA provides an accurate and rapid means of detecting HAV in fecal specimens. The test was found to be as sensitive as SPRIA and almost as sensitive as IEM, which is in accord with reports of this system for detection of the human rotavirus in fecal specimens (17) and hepatitis B surface antigen in human sera (15).

IEM, although sensitive, is less readily applied to large-scale detection of virus than immune adherence hemagglutination (9, 11) and SPRIA (4, 14). However, the latter tests require virus to be partially purified, usually by isopycnic banding in CsCl, as a preliminary to testing, a step which is time consuming and restricts the number of specimens which can be processed. On the other hand, the ELISA and SPRIA tests described here will detect HAV in comparatively crude fecal samples.

The limited availability of monospecific animal antisera to HAV has necessitated the use of human sera in most serological tests. Since human sera contain antibody to a broad range of antigens, including bacterial and viral antigens commonly found in feces, IEM, SPRIA, and ELISA tests for HAV will detect not only HAV but any other antigens that the host's immune system has been previously primed against. Therefore, the use of pre- and postinfection hepatitis A sera in the IEM test and the blocking and neutralization tests of the ELISA and SPRIA methods are essential to assess the specificity of any positive results.

Although the complete ELISA test took 30 h to perform, the sensitivity of the test was not appreciably altered by reducing the incubation time to 4 h (2 h for virus, 2 h for blocking), which enabled samples to be tested the day after collection.

In our system the sensitivity of the ELISA test may have been set rather low by the choice of very strict criteria for the specific blocking reaction. It is probable that these criteria could be relaxed if the results were read with a spectrophotometer.

It is likely that the ELISA test will find considerable clinical application, since it permits the rapid screening of large numbers of specimens

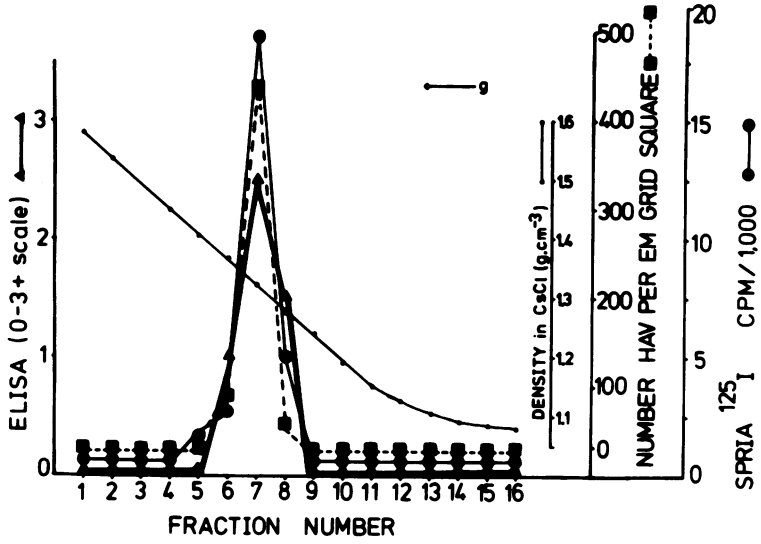

FIG. 3. *Detection of HAV by IEM (■), SPRIA (●), and ELISA (▲), after isopycnic ultracentrifugation of specimen 1 (Table 1) in a linear CsCl density gradient.*

by laboratories which do not have access to equipment such as an ultracentrifuge or an electron microscope. Also, ELISA screening of fecal specimens will become very useful as a preliminary step to IEM screening for detecting large quantities of HAV for use as reagent antigen. Furthermore, the antibody-coated plates can be stored at 4°C for up to 1 month without loss of activity, and the conjugate can be stored for several months provided it is kept frozen. These features, and its relatively low cost, make ELISA an ideal system for large-scale epidemiological studies.

Before a serological diagnosis of hepatitis A infection can be established, a period of 3 to 4 weeks between collection of paired sera is required to detect a significant rise in anti-HAV by IEM (8), SPRIA (6, 14), or immune adherence hemagglutination (9). Hepatitis A-specific IgM is currently detected after fractioning an acute-phase serum sample into its 19S and 7S components on sucrose density gradients (6), thereby limiting the value of this procedure for rapid, large-scale serodiagnosis. A diagnosis of hepatitis A infection was made in more than half of the 33 patients on the basis of detection of HAV in feces by IEM, SPRIA, or ELISA. In naturally acquired disease, fecal shedding of HAV has usually declined to undetectable levels after 1 week from the onset of dark urine (8). If fecal specimens are collected within 1 week from the onset of dark urine, detection of HAV could have some rapid diagnostic value, especially when results can now be obtained by ELISA 1 day after specimen collection. However, rapid and reliable diagnosis of hepatitis A infection seen later than 1 week after onset will require the development of a convenient and efficient assay for hepatitis A-specific IgM.

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ADDENDUM

Since the writing of this manuscript, a similar ELISA procedure for detection of HAV and anti-HAV has been published (L. R. Mathiesen, S. M. Feinstone, D. C. Wong, P. Skinhoej, and R. H. Purcell, J. Clin. Microbiol. 7:184-193, 1978).

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