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Comparison of Sensitivity of Radioimmunoassay and Immune Electron Microscopy for Detecting Hepatitis A Antigen in Fecal Extracts[1] (39772)

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Introduction. Although the relationship of hepatitis A antigen (HA Ag) to infectious, or short incubation, hepatitis is now fairly well-established (1-4), HA Ag for research purposes remains in short supply. Unlike hepatitis B antigen (HB_sAg), which can be recovered in large quantities from the plasma of carriers, HA Ag, when present at all, probably exists in plasma in very low titer. HA Ag is more easily recovered from the feces of infected patients, but then only during a rather restricted period, which generally terminates before the appearance of the clinical symptoms of disease (4, 5). Thus, the probability of the recovery of substantial quantities of hepatitis A antigen from human patients would appear to be quite low.

During a 1970 epidemic of hepatitis A at the Lynchburg (Virginia) Training School and Hospital, Matthew *et al.* (6) reported 375 clinical and 190 subclinical cases of hepatitis A among 3600 patients in the institution. Several fecal samples collected during that epidemic have been studied by immune electron microscopy (IEM) in an effort to determine the distribution of HA Ag during the course of the disease, and to find a source of antigen for research purposes. In some of the stools studied, typical 27-nm virus-like particles were found which reacted with antibody to hepatitis A (anti-HAV) in convalescent serum to form antigen-antibody complexes recognizable by electron microscopy (1). However, since the identification of complexes by the IEM technique requires the skill and experience of a trained electron microscopist, as well as the investment of large amounts of time to examine the grids, it seemed advisable to investigate more efficient techniques for large-scale sample screening.

The recent publications by Hollinger *et al.* (7) and Purcell *et al.* (8) demonstrated the feasibility of radioimmunoassay (RIA) for detecting HA Ag in density gradients and chimpanzee serum and stool samples. Although the RIA technique appeared to be as sensitive as IEM for the detection of HA Ag under these controlled conditions, little information was available to indicate its sensitivity using crude fecal suspensions. We have, therefore, examined feces collected during various stages of an hepatitis A epidemic to determine the usefulness of RIA for detecting HA Ag in relatively crude clinical specimens. The following report documents our findings.

Materials and methods. Fecal samples. Four hundred and sixty-three fecal samples were collected in 1970 from wards in which clinical cases of hepatitis occurred (6). These samples have been stored at −70° since that time. For the present study, 63 stools were selected: 28 were from patients who developed clinical hepatitis, 19 from patients who developed subclinical disease, and 16 from patients who remained asymptomatic throughout the epidemic. A diagnosis of clinical hepatitis was based upon development of clinical signs, i.e., fever, malaise, diarrhea, dark urine, and jaundice, and was confirmed by demonstrating bilirubinemia and elevated serum glutamic pyruvic transaminase (SGPT). A diagnosis of subclinical hepatitis was made if the SGPT level in the weekly serum samples rose

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above 50 units. Those patients whose weekly SGPT levels never excceded 50 units were considered to be asymptomatic, but not necessarily uninfected.

Based on conclusions from previous reports (4, 5), stools from patients with hepatitis were selected primarily from among those collected over a 3-week period prior to peak transaminase elevation. As a check on methodology, several fecal suspensions were duplicated under code. Thus, we report results on 77 samples.

A 5% fecal suspension was prepared using veal infusion broth adjusted to pH 8.5 with 0.05 *M* borate buffer containing 0.05% polyethylene oxide (9). The raw stools were weighed in small plastic bags. After the broth was added, they were homogenized for 2–3 min in a "Stomacher-80" (Cooke Laboratory Products, Division of Dynatech Laboratories, Alexandria, Va.). The suspension was centrifuged at 1000*g* for 10 min; the resulting supernatant was again centrifuged at 10,000*g* for 10 min. This supernatant was carefully removed and stored at −20°.

Immune electron microscopy. IEM was performed as previously described by Feinstone *et al.* (1). Briefly, serum from a patient who had recovered from hepatitis A infection was used as a source of antibody. This serum had a titer of approximately 1:32,600 by immune adherence hemagglutination assay (IAHA) (10). To 0.9 ml of a 5% fecal suspension, 0.1 ml of a 1:10 or 1:100 dilution of this serum was added and incubated overnight at room temperature. The suspension was centrifuged at 40,000 rpm in a Ti-50 rotor for 30 min and the resultant pellet was suspended in 0.1 ml of deionized water. A drop of the suspension was placed on a 400-mesh carbon-coated grid, allowed to stand for 2 min and then drawn off. Thereafter the residue was fixed with 3% glutaraldehyde and stained with 3% phosphotungstic acid. The grids were examined in the electron microscope (as coded samples) for the presence of HA antigen-antibody complexes. Three good grids of each sample were thoroughly examined before a suspension was judged positive or negative.

Radioimmunoassay. RIA tests were performed in two laboratories as randomized coded samples. In the Bethesda laboratory (RIA-ENLI) 100 μl of purified γ-globulin (80 μg/ml) from serum known to contain anti-HA (same source as used for IEM) were added to each well of a polyvinyl microtiter "U" plate (Cooke Laboratory Products) and incubated at room temperature in a moist chamber for 3 hr, then at 4° for 15–18 hr. The wells were washed with a saline buffer containing 2% fetal calf serum. Fifty microliters of each sample to be tested were added to two noncontiguous microtiter wells and the plates were incubated at 37° for 18 hr. The samples were removed and the wells were thoroughly rinsed with saline buffer. Finally, 75 μl of ^{125}I-labeled IgG (approximately 200,000 cpm) were added to each well and incubated for 4 hr at 45°. The γ-globulin had been prepared on a DEAE-cellulose column (11) using serum taken from the same patient referred to above. The wells were washed six times with buffer, cut out, and the radioactivity was determined. A fecal sample taken from a donor with no previous history of hepatitis was used as a negative control. Samples were considered positive if the ratio of the counts per minute of the sample (P) divided by the counts per minute of the negative (N) was greater than 3.

The Arizona laboratory (RIA-AZ) used a modification of the test system previously described (7). Briefly, 200 μl (1–2 μg) of chromatographically purified IgG containing anti-HAV activity were used to coat the wells of a polyvinyl microtiter "U" plate (220-24, Cooke Engineering Co., Alexandria, Va.). The plate was covered with parafilm and incubated for 3 hr at room temperature, then overnight at 4° 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). Fifty microliters of the sample to be tested for HAV were added to each of two wells and the plate was incubated by flotation in a water bath at 45° for 2 hr. The wells were then aspirated by suction and washed five times with PBS-FCS. After washing, 50 μl (300,000 cpm) of ^{125}I-labeled and chromatographically pur-

ified IgG containing anti-HAV activity were added to each well, and the plate was incubated for 90 min at 45°. After an additional washing step to remove unbound labeled antibody, individual wells were cut out and counted in a γ counter for a total of 4000 counts. The residual mean cpm of at least eight control samples devoid of HAV were used to determine the mean background cpm.

Results. A summary of the results obtained by the two RIA tests and by IEM in screening crude fecal extracts for HA Ag (HAV) is presented in Table I. Of 77 samples tested, 61 (79%) gave the same results by all three assay methods: 28 were positive by both RIA and IEM and 33 were negative. There were 14 samples positive by at least one RIA method which were negative by IEM.

The correlation between IEM positivity and RIA was very strong. By IEM, 30 samples (39%) contained detectable antigen–antibody complexes. All but two of these were likewise positive by both radioimmunoassays; the remaining two were positive by one RIA. From among the clinical and subclinical groups, RIA-AZ detected as positive five samples that RIA-ENLI did not; RIA-ENLI detected one positive sample that RIA-AZ did not. All of these were negative by IEM. Among other samples negative by IEM were five collected from asymptomatic patients which were positive by RIA-AZ. Two of these were also positive by RIA-ENLI.

TABLE I. RESULTS OF SCREENING FOR HEPATITIS A ANTIGEN BY RADIOIMMUNOASSAY AND IMMUNE ELECTRON MICROSCOPY.

Group of patients[a]	Fecal samples tested	RIA-AZ[b]	RIA-ENLI[c]	IEM-ENLI
Clinical hepatitis (41)	19	+	+	+
	3	+	+	–
	4	+	–	–
	1	+	–	+
	1	–	+	+
	1	–	+	–
	12	–	–	–
Subclinical hepatitis (20)	9	+	+	+
	1	+	–	–
	10	–	–	–
Asymptomatic (16)	11	–	–	–
	2	+	+	–
	3	+	–	–

[a] Includes duplicates among the coded samples.

[b] A sample was considered positive if the radioactivity was greater than 1000 cpm (approximately $3 \times P/N$).

[c] A sample was considered positive if the ratio of the cpm of the sample (P) to the cpm of a negative control (N) was greater than 3.

From the data presented in Table I, it is evident that HA Ag was detected by all three systems in about the same percentage of patients with subclinical hepatitis as with clinical disease.

The pattern of occurrence of fecal HA Ag varied somewhat from patient to patient. It was detected as early as 21 days before peak liver enzyme elevation (serum glutamic pyruvic transaminase; SGPT) and as late as 14 days after peak elevation. In general, maximum HA Ag activity, as determined both by radioactivity and particle counts, occurred from 15 to 5 days before peak SGPT levels.

The relative sensitivities of IEM and RIA-ENLI were further compared by examining a series of twofold dilutions of a fecal extract known to contain HA Ag. Fifty microliters of sample were used for each RIA-ENLI determination and 900 μl were used for each IEM determination. The data are summarized in Table II. For comparison, a fecal extract negative for HA Ag was also run serially. To determine the positive/negative ratio, a control was used consisting of phosphate-buffered saline containing 2% fetal calf serum (PBS + 2% FCS).

In this series of samples by IEM it was possible to find with relative ease individual virus particles complexed with antibody up to a dilution of 1:64. Thereafter, complexes could be found, but generally only after extensive EM examination of grids. Samples tested by RIA were still positive at a fecal dilution of 1:256. The negative controls were consistently negative by IEM and RIA.

An indication of the specificity of the RIA is shown in Table III. In addition to the Lynchburg stool extracts, eight coded samples were included in the panel which were not from Lynchburg. This group was composed of duplicates of a 5% extract used as a

TABLE II. COMPARISON OF SENSITIVITY OF RIA AND IEM TECHNIQUES FOR DETECTING HEPATITIS A ANTIGEN IN SERIAL DILUTIONS OF HA Ag-POSITIVE FECAL EXTRACTS.

	Radioimmunoassay				Immune Electron Microscopy	
	HA Ag Positive		HA Ag Negative			
Dilutions of 5% fecal extract	cpm[a]	Ratio[b]	cpm	Ratio	HA Ag Positive (Particles per grid square[c])	HA Ag Negative (Particles per grid square)
1:2	65305	159.67	732	1.79	138	0
1:4	48222	117.90	623	1.52	75	0
1:8	27914	68.24	604	1.48	58	0
1:16	14127	34.54	534	1.30	24	NT[d]
1:32	6813	16.66	505	1.23	10	NT
1:64	3445	8.42	471	1.15	3	NT
1:128	1995	4.88	421	1.03	1	NT
1:256	1299	3.18	484	1.18	1	NT
1:512	672	1.64	410	1.00	1	NT
1:1024	694	1.70	416	1.02	NT	NT
1:2048	531	1.30	531	1.30	NT	NT
1:4096	520	1.27	487	1.19	NT	NT
PBS + 2% FCS	409	1.00				

[a] Counts per minute (cpm) given as average of two microtiter wells per determination. The sample volume was 0.05 ml.

[b] The ratio = counts per minute of test sample/counts per minute of control (PBS + 2% FCS).

[c] Particles per grid square given as average of counts from five grid squares. The sample volume used for the preparation was 0.9 ml of 5% fecal extract plus 0.1 ml of convalescent serum (1:100).

[d] NT, not tested.

TABLE III. EFFECT OF EXOGENOUS ENTEROVIRUS ON SPECIFICITY OF RADIOIMMUNOASSAY FOR HEPATITIS A ANTIGEN.

	RIA (cpm)[a]	
Sample	Unknown 1	Unknown 2
Negative control (5%)	797[a]	770
Negative control + polio[b]	792	779
Negative control + LF108tc[c]	764	734
Negative control + A-2 plaque virus[d]	754	723

[a] Each value is the average of two determinations.

[b] Approximately 10^{10} virus particles per milliliter were added to each extract.

[c] Entero-type virus isolated from Lynchburg fecal sample 108 collected from patient with subclinical hepatitis; identified as Coxsackie A24.

[d] Originally isolated at the Armed Forces Institute of Pathology by A. D. Felsenfeld from icteric phase sera of military patients with documented diagnoses of viral hepatitis.

negative control, plus duplicates of this same negative sample containing one of three entero-type viruses: polio; A-2 plaque virus (12); and LF108tc, isolated from a stool collected from a Lynchburg patient with subclinical hepatitis. Each value in Table III is the average of two RIA determinations. It is clear that the addition of the viruses in no way interferes with the specificity of the assay, and that the radioactivity of the entero-type-virus-containing extracts is essentially the same as the virus-free control.

Further evidence of the specificity of the RIA is presented in Table IV, wherein a comparison is made between the binding of ^{125}I-labeled IgG from convalescent human (RIA-ENLI) and chimpanzee (RIA-AZ) sources and the binding from human IgG sources (RIA*-ENLI) negative by IAHA for antibody to HA. Strongly positive samples (9–15) have high radioactivity when ^{125}I-labeled IgG containing anti-HA is used and low radioactivity with anti-HA-negative human ^{125}I-labeled IgG. Most negative samples (as exemplified by samples 6 and 7) have low radioactivity with each ^{125}I-labeled IgG. The exceptions are samples 1 to 5. The high radioactive responses of samples 4 and 5 in all three tests suggest that these samples are false positives. The positive reaction of samples 1 to 3 in RIA-AZ may be due to an antihuman component in the chimpanzee's

TABLE IV. COMPARISON OF RESULTS OF RADIOIMMUNOASSAYS FOR PRESENCE OF HA Ag USING ^{125}I-LABELED IgG FROM A CONVALESCENT CHIMPANZEE (RIA-AZ), A CONVALESCENT HUMAN (RIA-ENLI), AND A POOL OF HUMAN SERA NEGATIVE FOR ANTI-HA (RIA*-ENLI).

Sample number	RIA-AZ (cpm)	RIA-ENLI (cpm)	RIA*-ENLI (cpm)	IEM-ENLI[a]	Disease State[b]
1	3254	90	74	−	A
2	1548	158	123	−	A
3	2287	446	371	−	A
4	3928	2870	3562	−	A
5	658	1292	983	−	A
6	385	318	287	−	A
7	103	182	131	−	C
8	1310	896	49	−	SC
9	8800	9761	57	+	C
10	4524	11978	235	+	SC
11	4887	6147	322	+	SC
12	9431	8077	65	+	C
13	8773	14107	101	+	SC
14	9059	7313	145	+	C
15	10602	9052	66	+	C

[a] Samples positive and negative by IEM for presence of antibody–antigen complexes are indicated by + and −, respectively.

[b] A, asymptomatic; C, clinical hepatitis; SC, subclinical hepatitis.

IgG, since the reaction does not occur with either human ^{125}I-labeled IgG.

Discussion. The usefulness and reliability of the RIA method for detection of HA Ag in crude fecal extracts is demonstrated in this report. Our studies agree with those of Hollinger *et al.* (7) and Purcell *et al.* (8) that γ-globulin from human serum containing high titer antibody to hepatitis A antigen can be labeled with ^{125}I and successfully used in an RIA solid phase "sandwich" technique to detect HA Ag, even in a very heterogeneous medium. The RIA procedure requires much fewer samples and less work-time per sample than does IEM, and is capable of more readily detecting lower concentrations of antigen. Purcell *et al.* (8) reported positive results with their micro-SPRIA system on acute phase fecal samples in which particles could not be visualized by IEM. They speculated that these results might be due to false positive reactions or nonparticulate HA Ag. These same results could also be explained by the greater sensitivity of RIA, especially when one considers the exhaustive search often required, and the role played by chance, to find low titer HA antigen by IEM in crude stool suspensions.

The most puzzling data in the present study are the five IEM-negative stool specimens collected from asymptomatic patients that were positive by RIA-AZ. By using ^{125}I-labeled IgG negative for anti-HA in combination with ^{125}I-labeled IgG positive for anti-HA, it was possible to show that the two samples also positive by RIA-ENLI owed their apparent positive reactions to components not associated with hepatitis antigen. The positive reactions of the three samples positive only with the chimpanzee ^{125}I-labeled IgG may be due to a nonspecific antihuman response in the animal, since the two human reagents were not positively reactive.

Subsequent to this comparative study, several hundred primate and human fecal extracts have been assayed by RIA-ENLI for the presence of HA Ag. Of this number, approximately 8% have produced false positive reactions. Further work is in progress to determine the basis of these RIA-positive responses.

Dienstag *et al.* (4) reported an epidemic of hepatitis A among naval personnel at San Diego and found 27-nm virus-like particles only in patients with icteric hepatitis, and then only up to the time of peak transaminase. This was not the case with the Lynchburg patients. Thus far, approximately identical percentages of patients with clinical and subclinical hepatitis have been found to excrete HA Ag in their feces. In fact, the stool sample used to generate the data for

Table II in the present report had the highest concentration of virus particles of any sample tested. It was collected from a subclinical case of hepatitis A 2 weeks after peak transaminase elevation.

In spite of the relatively high rate of false positives, RIA offers several distinct advantages over the more tedious and time-consuming IEM method. RIA is simpler, more conservative of valuable reagents, more sensitive, and more rapid, especially for screening large numbers of crude fecal specimens for HA Ag. In our experience, an IEM-positive sample will also be positive by RIA. Thus, RIA screening conveniently selects out the most likely sources of antigen. The rare fecal sample which contains antigen sufficient for research purposes will be unmistakably positive by RIA.

Summary. Solid-phase, microtiter radioimmunoassay (RIA) has been compared with immune electron microscopy (IEM) for its sensitivity and usefulness in detecting hepatitis A antigen in clinical fecal specimens. Using relatively crude 5% extracts from stools collected at the Lynchburg Training School and Hospital in 1970 during an epidemic of infectious hepatitis, we have demonstrated the feasibility of RIA as a rapid and reliable screening test. All samples positive for HA Ag by IEM were also positive by RIA. Dilution experiments with HA Ag-positive fecal preparations indicated that RIA is at least as sensitive as IEM. RIA is an appropriate method for reliable, large-scale screening of fecal samples for HA Ag. RIA would appear to be potentially useful in the diagnosis of hepatitis A infection.

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