A radioimmunoassay for hepatitis A antigen and antibody

S. J. SKIDMORE AND E. H. BOXALL

From the Regional Virus Laboratory, East Birmingham Hospital, Bordesley Green, Birmingham B9 5ST, UK

SUMMARY The HAVAB radioimmunoassay for the detection of antibody to hepatitis A is assessed. Its modification to detect virus in faecal samples is described and in this it was found to be more sensitive and specific than electron microscopy.

Progress in the field of hepatitis A research has been hindered by a lack of standard reagents, mainly the difficulty of finding convenient sources of virus. In spite of this, several tests for virus and antibody have been described by various workers. These include immune electron microscopy, the test used successfully in finding the causative agent in faeces from patients with hepatitis A (Feinstone *et al.*, 1973) and complement fixation and immune adherence haemagglutination, which were developed in an attempt to provide a more sensitive and less laborious diagnostic test (Krugman *et al.*, 1975). However, radioimmunoassay promises to be the most useful as it is relatively quick and easy to perform and is very sensitive (Hollinger *et al.*, 1975; Purcell *et al.*, 1976).

We have been trying for some time to develop a diagnostic test for this infection; at first we used immune electron microscopy. In samples from some of our patients small virus particles resembling those of hepatitis A and reacting with anti-MSI serum obtained from the National Institutes of Health have been observed and described (Skidmore and Boxall, 1977). However, the interpretation of results has been particularly difficult since stool samples often contain many types of small particles, and no hepatitis A virus has been available for reference.

Recently, however, Abbott Laboratories have introduced a commercial radioimmunoassay kit for the detection of antibody to hepatitis A. This has provided us with an opportunity to study samples from our 'infectious hepatitis' patients using standard reagents.

Methods

ANTIBODY TESTS

The tests for antibody to hepatitis A (anti-HAV)

were all done using the HAVAB radioimmunoassay (RIA) kit supplied by Abbott Laboratories (Abbott Park, North Chicago, Illinois 60064, USA). This is a solid-phase, competitive RIA using beads coated with hepatitis A antigen. Of each sample to be tested for antibody, 10 μ l were dispensed into trays provided with the kit; 200 μ l of 125 I-labelled anti-HAV were added to each well, and one antigen-coated bead was dispensed into each well. The reaction trays were covered and incubated at room temperature for 24 hours. Then the beads were washed thrice in distilled water and counted for 60 seconds in a Nuclear Enterprises 1600 gamma counter.

The net count rate of each specimen is compared to a cut-off value to determine whether antibody is present. The cut-off value is calculated by dividing the sum of the mean negative control and the mean positive control by two. Specimens with counts per minute (cpm) values greater than the cut-off value are negative and vice versa.

ANTIGEN TESTS

The test described above was modified to provide a radioimmunoassay to detect antigen, using the same reagents and basing the method on that of Purcell *et al.* (1976). The wells of polyvinyl microtitre plates (Linbro Chemical Co Inc, New Haven, Connecticut, USA) were coated with a 1:100 dilution in saline of either the positive or negative control sera taken from the HAVAB kit. The plates were used in parallel so that a non-specific result could be identified by a reaction in both plates, whereas a specific positive reaction would be detected only in the anti-HAV coated plate. An antiserum with a high level of antibody to hepatitis A, as measured by the HAVAB test, could also be used. After 4 hours at 4°C the plates were washed, coated with 1% bovine serum albumin (BSA), and left at 4°C overnight. The plates were then ready to be

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used. For the test, a 15% (v/v) suspension of faeces in saline was centrifuged at approximately 3000 rev/min in an MSE Superminor centrifuge and the supernatant was harvested; 25- μ l volumes of this supernatant were placed in duplicate wells and the plates were incubated at 4°C for approximately 66 hours. The plates were washed with saline, and 50 μ l of 125 I-labelled anti-HAV were added to each well; the plates were then incubated for 5 hours at 37°C. After this time the plates were again washed and cut apart with scissors, each well being counted as described above. Each sample of faeces was tested in both the positive and negative plates, initially using BSA as a negative control but later using a faecal extract which had been tested and shown to be negative.

NEUTRALISATION (ANTIBODY-BLOCKING) STUDIES

These were carried out basically as described above using a known positive faecal extract as the standard antigen. After incubation 25 μ l of neutralising serum were added to the appropriate wells. Saline was used in control wells. The plates were left at 37°C for 1 hour before 25 μ l of 125 I-anti-HAV was added for a further 5 hours. The wells were then washed and counted as described previously.

CAESIUM-CHLORIDE DENSITY CENTRIFUGATION

For buoyant density determinations, faecal samples had to be further concentrated after the initial low-speed centrifugation (Skidmore and Boxall, 1977). The supernate from this spin was centrifuged at 7000 rev/min for 30 minutes in an MSE super-speed 65 centrifuge to deposit cellular debris, and the supernate from this second spin was then centrifuged at 50 000 rev/min for 2 hours. The pellet was resuspended in 1 ml of phosphate-buffered saline (PBS) and layered onto 4 ml of caesium chloride, density 1.4 (62.5% w/v). The gradient was centrifuged at 40 000 rev/min for 18 hours, and 0.2 ml fractions were collected from the bottom of the tube.

ELECTRON MICROSCOPY

Material to be examined by electron microscopy was applied to carbon-formvar coated grids. After drying, the grids were washed three times in distilled water and negatively stained with potassium phosphotungstate (2% w/v, pH 5.5).

Results

ANTIBODY TESTS

Assays for antibody were carried out as described

above and determined positive or negative by the criteria suggested in the kit. When we tested single sera from hepatitis B negative patients with suspected viral hepatitis we found that 78% (227/290) contained antibody to HAV. Only 36% (31/86) of sera from healthy 'controls' were positive. The groups were similar in age, sex, and socioeconomic level. Since the proportion of positives in the control group remains the same even though the total number of samples tested is continually increasing, we feel that the result represents the level of antibody in the general population.

In addition to the information that a serum sample was positive or negative, we needed a value for each serum that would represent the amount of antibody present. In competitive RIAs the amount of radioactivity-labelled antibody binding to the bead is inversely proportional to the amount of anti-HAV in the test sample, assuming that the reaction reaches equilibrium under the conditions of our test (24 hours at room temperature). This can be expressed as a count ratio, which is simply the cpm in the negative control/the cpm in the test sample.

We hoped to find high count ratios in patients with obvious recent infections, but, in fact, there was no consistent difference in count ratios between sera from patients with recent hepatitis and the 'controls' (patients with no history of recent hepatitis). Both groups showed a wide range of values.

In addition to giving an indication of the amount of antibody present, the count ratio enabled us to compare different batches of assays and also repeated tests on the same sample as it is not possible to compare radioactive counts directly. Of 25 samples tested twice, usually with a different batch of the assay, in only four cases was there a difference of more than one unit between the values obtained.

In 17 paired sera from patients suspected of having non-B viral hepatitis and tested in parallel (Table 1), eight showed an increase in antibody level as expressed as a count ratio, although the first sera of two of these already had a high count ratio (Nos 2 and 17); three pairs were negative and six pairs were stationary.

From these results it seems that, to make a diagnosis by detecting antibody, paired sera in which a rising count ratio can be demonstrated will be needed. However, of the six pairs of sera classed as stationary, three second sera (Nos 4, 12, and 15) showed a small rise from an already high level.

In 10 paired sera from hepatitis B patients (Table 2), none showed any rise in count ratio, evidence for the specificity of the antibody response in hepatitis A patients.

Table 1 Results of antibody tests on paired sera from suspected hepatitis A patients

Pair No.		Counts (cpm)	Count ratio	Interval between serum samples (days)
1	† 1st	21903	1.1	24
	2nd	34224	0.7	
2	* 1st	1388	17.4	6
	2nd	953	25.4	
3	† 1st	1741	13.9	4
	2nd	1863	13.0	
4	† 1st	1184	20.4	12
	2nd	973	24.8	
5	* 1st	8291	2.9	25
	2nd	1087	22.2	
6	* 1st	3983	6.0	25
	2nd	1137	21.3	
7	† 1st	16849	1.4	4
	2nd	16820	1.4	
8	* 1st	4870	4.9	12
	2nd	1587	15.2	
9	* 1st	3082	7.8	12
	2nd	1907	12.1	
10	† 1st	1927	18.0	19
	2nd	1738	19.9	
11	* 1st	3793	9.1	42
	2nd	1955	17.7	
12	† 1st	2607	13.5	9
	2nd	2112	16.8	
13	† 1st	1396	24.8	12
	2nd	1511	23.0	
14	† 1st	30534	1.1	8
	2nd	25489	1.3	
15	† 1st	3797	9.1	14
	2nd	2677	12.9	
16	* 1st	4565	7.6	34
	2nd	1722	20.1	
17	* 1st	2432	14.2	45
	2nd	1689	20.5	

*Rise in titre

†Stationary

‡Negative

Table 2 Results of antibody tests on paired sera from hepatitis B patients

Pair No.		Counts (cpm)	Count ratio	Interval between serum samples (days)
1	1st	1640	9.4	71
	2nd	1924	8.0	
2	1st	1873	8.2	335
	2nd	1600	9.6	
3	1st	28653	0.5	27
	2nd	26703	0.5	
4	1st	1032	15.0	40
	2nd	1508	10.2	
5	1st	1345	11.5	53
	2nd	1124	13.7	
6	1st	1158	13.3	35
	2nd	1732	8.9	
7	1st	1334	11.6	70
	2nd	1639	9.4	
8	1st	13882	1.1	93
	2nd	4675	3.3	
9	1st	1222	12.6	66
	2nd	1451	10.6	
10	1st	13468	1.1	134
	2nd	16732	0.9	

MODIFICATION OF THE ANTIBODY TEST TO DETECT FAECAL ANTIGEN

We had been hampered in our attempts to set up a radioimmunoassay for the detection of antigen, using our own reagents, by an insufficient quantity of convalescent serum from which to obtain specific IgG. The availability, from a commercial source, of labelled anti-HAV antibody provided the opportunity to do this. We used the method described above.

Out of 65 faecal samples tested from jaundiced patients, 11 were positive. When nine of the samples positive by RIA were examined by electron microscopy, in only two could virus particles be found. The serum samples from six of the remaining 54 were also negative for antibody by RIA, and therefore these were probably not cases of hepatitis A.

Another 11 samples previously tested and found by electron microscopy to contain small virus particles similar to hepatitis A were all negative by RIA, stressing the greater specificity of the RIA. Thirteen samples from controls were all negative, while 5/8 contacts of a jaundiced case were positive, although only one showed any signs of illness. This emphasises the number of subclinical cases of hepatitis A that occur.

DETECTION OF HEPATITIS A ANTIGEN IN A CAESIUM CHLORIDE GRADIENT BY RIA

In a previous report we described the virus associated with hepatitis A as having a buoyant density of 1.34 in caesium chloride (Skidmore and Boxall, 1977). To check this result we carried out a similar experiment using four of our RIA-positive samples; 0.2 ml gradient fractions were collected and tested by RIA. The Figure shows the results of one sample. A peak of activity can be seen in fraction 11, which corresponds to a density of ≈ 1.35 . However, we also found activity at other densities, in this case

Figure Distribution of hepatitis A virus in a caesium chloride gradient. Virus was detected in gradient fractions by RIA.

1.45, which we had not detected by electron microscopy, presumably because of a lack of sensitivity.

SPECIFICITY OF THE ANTIBODY AND ANTIGEN TEST

To test the specificity of this reaction we carried out neutralisation tests as described in Methods.

Using two sera (RL and J McG), which were found to have high levels of antibody by the HAVAB test, we obtained almost complete neutralisation of the standard antigen as shown by an 85% and an 80% reduction, respectively, in the number of counts (Table 3). A negative serum (TJ) gave no significant neutralisation.

Table 3 *Specificity of the radioimmunoassay results showing the blocking of attachment of ^{125}I -labelled antibody by various sera*

Neutralising serum	Counts (average of 3 values)	% Reduction
None	1020	—
RL	149	85.4
J McG	196	80.8
TJ	957	6.2
None	638	—
Pre- LSH	655	0
Post- Chimp	123	80.8
Pre- NIAID	941	0
Post- Chimp	133	79.2

Similar results were obtained using two sets of paired chimpanzee sera: one set supplied by Professor A. Zuckerman, of the London School of Hygiene (LSH), and the other by the Research Resources Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIAID), Bethesda, Maryland, USA. In both cases pre-infection serum did not 'block' the ^{125}I -labelled antibody from reacting with the known antigen, whereas the convalescent serum reduced the counts to those of the control well.

Discussion

The introduction of a commercially produced kit for the detection of antibody to hepatitis A is a major advance in the diagnosis of this infection.

We used the kit to look at serum samples from jaundiced patients and found that 78% had some level of antibody. It seemed unlikely that such a large proportion of jaundiced, non-hepatitis B patients would have hepatitis A, and as 36% of control sera have anti-HAV activity it is not unreasonable to expect that a similar proportion of jaundiced patients have antibody as a result of past infection. If this, together with cases where the final diagnosis is not hepatitis, is taken into consideration, then up

to 50% of non-B hepatitis among our cases may be due to hepatitis A.

The high percentage of positive samples made it economically and practically difficult to titrate them all, and we therefore used a count ratio to indicate the amount of antibody present. As expected, this varied greatly from sample to sample, and we concluded that the presence of a high level of antibody does not, by itself, indicate a recent infection.

Paired sera showing a significant rise in count ratio seem to be the best serological diagnostic test, as is the case with other viral infections. We showed that some paired sera taken from patients in an outbreak of hepatitis A showed a distinct rise in titre associated with recent infection. In contrast, paired sera taken from patients suffering from hepatitis B acquired as a result of acupuncture (Boxall, 1978) showed no change in count ratio, thereby proving the specificity of the rise. However, even when a serum sample is taken soon after the onset of illness it may already have a high titre of specific antibody. This is because during the course of a hepatitis A infection antigen is present and being excreted before definitive symptoms appear (Dienstag *et al.*, 1975). Therefore a high stationary titre may be suggestive of a recent infection, although it is not truly diagnostic. Another problem is that for several reasons a second serum sample is often unavailable, and in these cases the detection of specific IgM may be the only serological way to provide a diagnosis of recent infection. Several groups of workers, including ourselves, have preliminary findings on such a test using either ELISA or RIA and the results look promising (Frosner *et al.*, personal communication 1978; Duermeier and van der Veen, 1978).

All serological diagnoses by rising titre of specific antibody are, by definition, retrospective, and we felt that such results would be of epidemiological interest only and of no direct use to the clinician in the management of his patient.

Our previous work had been aimed at developing a rapid diagnostic test for hepatitis A based on the detection of viral antigen in the faeces (Skidmore and Tadros, 1976; Skidmore and Boxall, 1977). As a result of our investigations we had several series of faecal material which we were eager to investigate fully. By modifying the Abbott Laboratories antibody kit we were able to do this.

We found the RIA more specific than electron microscopy in that previously we had found by CsCl_2 density centrifugation, combined with electron microscopy, small virus-like particles in faeces from jaundiced patients which ultimately proved to be negative by RIA. The RIA test was also more sensitive, and we were able to detect antigen in

samples that were negative by electron microscopy. This agrees with the findings of Hall *et al.* (1977) and is particularly useful when testing contacts who may have a subclinical infection. In addition, because RIA does not involve the lengthy concentration procedures required for electron microscopy, we can now screen up to 100 samples a week, a 10-fold increase. More important is the fact that we have a test which automatically relates the antigen we find to the original MS-1 strain of hepatitis A described by Krugman *et al.* (1959). We were able to confirm by this method that the viruses we described previously (Skidmore and Boxall, 1977) were serologically similar to MS-1 and that their density was 1.34 in caesium chloride. However, we have found by RIA minor peaks of antigen at other densities, which we had not detected by electron microscopy, presumably because of a lack of sensitivity. The detection by RIA of minor peaks of activity is consistent with the findings of other workers (Bradley *et al.*, 1977), although whether these represent activity due to complete virus particles is as yet unknown.

Unfortunately, even with the introduction of this sensitive test, we still have the problem of getting early faecal samples; the number of virus particles excreted falls off so rapidly as clinical jaundice develops that even RIA cannot detect them. We found this in a patient whose faecal sample was RIA positive two days after, but negative five days after the onset of jaundice. We did demonstrate a rising level of anti-HAV in this man, so that in cases where patients clinically have all the symptoms of hepatitis A, yet prove to be negative for antigen, results from paired sera should provide a diagnosis. This leaves a number of jaundiced patients who fit into the category of non-A, non-B hepatitis. We have been studying jaundiced patients in one such group. They are all haemophiliacs and regularly receive blood products (Skidmore and Boxall, in preparation). Whether non-A, non-B hepatitis is due to a completely new infectious agent or whether it is a manifestation of a known virus is open to conjecture. With improved methods for diagnosing hepatitis A now available, the characterisation of all types of hepatitis will be facilitated.

References

- Boxall, E. H. (1978). Acupuncture hepatitis in the West Midlands, 1977. *Journal of Medical Virology*, **2**, 377-379.
- Bradley, D. W., McCaustland, K. A., Schreeder, M. T., Cook, E. H., Gravelle, C. R., and Maynard, J. E. (1977). Multiple buoyant densities of hepatitis A virus in caesium chloride gradients. *Journal of Medical Virology*, **1**, 219-226.
- Dienstag, J. L., Feinstone, S. M., Kapikian, A. Z., Purcell, R. H., Boggs, J. D., and Conrad, M. E. (1975). Faecal shedding of hepatitis-A antigen. *Lancet*, **1**, 765-767.
- Duermeyer, W., and van der Veen, J. (1978). Specific detection of IgM-antibodies by ELISA, applied in hepatitis A (Letter). *Lancet*, **2**, 684-685.
- Feinstone, S. M., Kapikian, A. Z., and Purcell, R. H. (1973). Hepatitis A: detection by immune electron microscopy of a virus-like antigen associated with acute illness. *Science*, **182**, 1026-1028.
- Hall, W. T., Bradley, D. W., Madden, D. L., Zimmerman, D. H., and Brandt, D. E. L. (1977). Comparison of sensitivity of radioimmunoassay and immune electron microscopy for detecting hepatitis A antigen in fecal extracts. *Proceedings of the Society for Experimental Biology and Medicine*, **155**, 193-198.
- Hollinger, F. B., Bradley, D. W., Maynard, J. E., Dreesman, G. R., and Melnick, J. L. (1975). Detection of hepatitis A viral antigen by radioimmunoassay. *Journal of Immunology*, **115**, 1464-1466.
- Krugman, S., Friedman, H., and Lattimer, C. (1975). Viral hepatitis, type A: identification by specific complement fixation and immune adherence tests. *New England Journal of Medicine*, **292**, 1141-1143.
- Krugman, S., Ward, R., Giles, J. P., Bodansky, O., and Jacobs, A. M. (1959). Infectious hepatitis. Detection of virus during the incubation period and in clinically inapparent infection. *New England Journal of Medicine*, **261**, 729-734.
- Purcell, R. H., Wong, D. C., Moritsugu, Y., Dienstag, J. L., Routenberg, J. A., and Boggs, J. D. (1976). A microtiter solid-phase radioimmunoassay for hepatitis A antigen and antibody. *Journal of Immunology*, **116**, 349-356.
- Skidmore, S. J., and Boxall, E. H. (1977). Small virus particles in faeces of patients with infectious hepatitis (hepatitis A). *Journal of Medical Microbiology*, **10**, 43-48.
- Skidmore, S. J., and Tadros, F. (1976). Hepatitis A virus (Letter). *Lancet*, **2**, 848-849.

Requests for reprints to: Dr Susan J. Skidmore, Regional Virus Laboratory, East Birmingham Hospital, Bordesley Green East, Birmingham B9 5ST, UK.