Detection of Immunoglobulin M Antibodies to Hepatitis A Virus by Enzyme-Linked Immunosorbent Assay

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An enzyme-linked immunosorbent assay for the detection of immunoglobulin M (IgM) antibodies to hepatitis A virus is described. The test uses the principle of binding of IgM antibodies to anti-IgM-coated microtiter plates to determine whether the IgM antibodies attached have specificities for hepatitis A virus. In three patients with hepatitis type A followed up to 12 months, IgM antibodies to hepatitis A virus could be demonstrated from the onset of illness and during the following 2 to 3 months. When acute-phase sera from 48 patients with acute hepatitis were tested, IgM antibodies to hepatitis A virus could only be demonstrated in 18 patients previously classified as type A, whereas 30 patients with type B and non-A non-B hepatitis were negative. IgM antibodies to hepatitis A virus could not be demonstrated in 108 normal sera nor in 55 sera containing rheumatoid factor. These results indicate that the enzyme-linked immunosorbent assay for IgM antibodies to hepatitis A virus is useful in the serodiagnosis of acute hepatitis type A on a single serum sample taken during the acute phase of illness.

In 1973 Feinestone et al. (6) described the demonstration of hepatitis A virus (HAV) and antibody to HAV (anti-HAV) by immune electron microscopy. Since then, different methods, such as complement fixation (18), immune adherence hemagglutination (15, 16), solid-phase radioimmunoassay (SPRIA) (7, 19), and enzyme-linked immunosorbent assay (ELISA) (10, 12, 20), have been used to detect HAV and anti-HAV.

Immune adherence hemagglutination, SPRIA, and ELISA have all been used in the serodiagnosis of acute viral hepatitis type A, but they require testing of acute- and convalescent-phase sera for anti-HAV (4). We have therefore evaluated an ELISA for the detection of immunoglobulin M (IgM) anti-HAV based on the principle described by Duermeyer and van der Veen (5).

**MATERIALS AND METHODS**

**Patients.** In three patients with acute viral hepatitis type A, serial sera were collected and tested for anti-HAV and IgM anti-HAV. Acute-phase sera from a group of 48 patients with acute viral hepatitis previously serologically confirmed as type A (17 patients), type B (24 patients), type A + B (1 patient), and type non-A non-B (6 patients) (11) were tested for IgM anti-HAV.

Sera from 98 of 225 Danish blood donors, in which anti-HAV had previously been demonstrated by blocking ELISA, and from 10 donors negative for anti-HAV (13) were tested for IgM anti-HAV. In addition, 55 sera randomly selected among sera found positive for rheumatoid factor at the laboratory for Autoimmune Diseases, Statens Seruminstitut, were tested for anti-HAV, IgM anti-HAV, and IgM antibodies to rubella.

**Methods.** IgM anti-HAV was detected by ELISA performed in microtiter plates (Dynatech, catalog no. 1-220-25) (Fig. 1). The plates were precoated with 75 µl of rabbit anti-human IgM specific for µ chains (no. 10-091, Dako, Copenhagen), diluted 1:25,000 in phosphate-buffered saline (PBS) (pH 7.4) and incubated for 24 h at 4°C in a humidified box. The plates were washed three times in PBS with 0.05% Tween 20, filled with 1% bovine serum albumin in PBS, and incubated for another 24 h at 4°C. After an additional three washings in PBS-Tween 20, the plates could be stored at 4°C in a humidified box until use, normally within 1 week. The sera to be tested were diluted 10-fold from 10^{-2} to 10^{-6} in PBS-Tween 20 containing 1% of a pool of cord blood sera found negative for anti-HAV, and 25 µl of the dilution was added to the plates and incubated for 4 h at room temperature. The cord blood in the diluent diminished background reactivity due to attachment of IgG to the plates, but did not interfere with the attachment of IgM.

After the plates were washed an additional three times, 25 µl of a stool extract containing HAV was added, and the plates were incubated overnight at 4°C. After three washings with PBS-Tween 20, 50 µl of a peroxidase-conjugated IgG prepared from a human convalescent-phase serum as described previously (12) and diluted in 50% human serum was added, and the plates were incubated for 2 h at room temperature. The human serum was a pool from 34 blood donors found to be negative for anti-HAV. After five washings in PBS-Tween 20, 100 µl of freshly prepared enzyme substrate (40 mg of orthophenylene diamine + 20 µl of 30% peroxide in 100 ml of citrate buffer, pH 5.0) was added, and the plates were incubated for 30 min at room temperature in the dark. The reaction was
stopped by adding 75 μl of 2 M sulfuric acid, and after the addition of 75 μl of PBS, bringing the total volume to 250 μl, the extinction at 493 nm was measured in a spectrophotometer with a rapid sampling microcuvette (Gilford model 250).

All the plates included a positive control, and as negative control a serum positive for anti-HAV in high titer but negative for IgM anti-HAV was employed. In addition, serial sera taken 1, 2, 3, and 6 months after the onset of acute hepatitis type A were always included in one of the plates. A serum was considered to contain IgM anti-HAV if the extinction at 493 nm for the serum divided by the extinction at 493 nm for the negative control serum (P/N) was above 2.1. The exact titer was calculated by interpolation from the highest 10-fold dilution giving a P/N value above 2.1 to the following dilution giving a lower P/N value, using 2.1 as cutoff. The negative control serum gave an extinction at 493 nm of about 0.05, and the positive serum gave about 0.5.

Anti-HAV was measured in a competition ELISA (11) (Fig. 2), a modification of the originally described blocking ELISA (12). Rheumatoid factor was detected by the Rose-Waaler test, and IgM antibodies to rubella were detected by ELISA (Fig. 3) (20a).

**RESULTS**

The results of serial determinations of anti-HAV and IgM anti-HAV in three patients with
acute hepatitis type A are shown in Fig. 4. In all three patients a fourfold titer rise in anti-HAV could be detected by competition ELISA, but this required that the first serum be taken very early and the second serum rather late. In all three patients IgM anti-HAV could be detected during the acute phase. Two of the patients were still positive for IgM anti-HAV 2 months after the onset of jaundice, but all patients were negative when tested later than 3 months after the onset of jaundice.

To determine the value of the test in diagnosing hepatitis type A in patients with acute hepatitis, we tested the acute-phase sera from 48 patients with acute hepatitis previously classified as type A, B, or non-A non-B (Table 1) (11).

In all 17 patients classified as type A, as well as in the patient with both type A and B, IgM anti-HAV was demonstrated at a titer higher than 10^4. None of the 40 patients with hepatitis type B or non-A non-B showed IgM anti-HAV, although 8 of them were positive for anti-HAV.

The diagnostic sensitivity of the IgM anti-HAV test was further established by the failure to demonstrate IgM anti-HAV in sera from 98 of the 225 Danish blood donors previously found by blocking ELISA to contain anti-HAV, and

from 10 blood donors found to be negative for anti-HAV (12).

When testing a panel of 55 sera containing rheumatoid factor in titers from 1:40 to 1:5,120 by Rose-Waaler, we found that 39 contained anti-HAV, but none contained IgM anti-HAV. Forty-one of the sera gave a positive reaction when examined for IgM antibodies to rubella by ELISA, but in all these cases the activity could be absorbed with latex particles coated with heat-aggregated IgG. Thus the results were all false-positive, probably due to a combination of IgG antibodies to rubella and rheumatoid factor.

**DISCUSSION**

Serological confirmation of hepatitis type A has previously been established by testing for anti-HAV by immune adherence hemagglutination, SPRIA, or ELISA (4). To be diagnostic, this requires either testing dilutions of acute-phase serum by both immune adherence hemagglutination and SPRIA or ELISA (4, 11) or testing dilutions of paired acute- and convalescent-phase sera in one of the tests and demonstrating seroconversion or a fourfold titer rise (11). Frequently no such titer rise can be demonstrated by SPRIA or ELISA if the first serum is not taken very early during the disease and the second serum more than 4 weeks later (11, 17). It is therefore of great value to have a test for IgM anti-HAV for the serodiagnosis of acute hepatitis type A.

Separation of IgM and IgG by sucrose gradient centrifugation and subsequent testing for anti-HAV has been used previously, but the process is rather time consuming (9, 17).

Bradley et al. (2) have described a modification of their SPRIA for anti-HAV, so that IgM and IgG antibodies could be distinguished, but the principle has not been evaluated further.

We have found that our modification of the ELISA for detecting IgM anti-HAV, originally described by Duermeyer and van der Veen (5), is easy to perform and gives reproducible results.

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**Table 1. Detection of IgM anti-HAV by ELISA in acute-phase sera from 48 patients with acute hepatitis previously classified as type A, B, and non-A non-B**

<table>
<thead>
<tr>
<th>Hepatitis type</th>
<th>No. of patients</th>
<th>Anti-HAV positive</th>
<th>IgM anti-HAV positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>17</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>B</td>
<td>24</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>A + B</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Non-A non-B</td>
<td>6</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

a All positive sera titrated out to >10^-6.
In testing a group of patients with acute hepatitis serologically confirmed as type A, B, or non-A, non-B, the ELISA for IgM anti-HAV showed a high accuracy for both positive and negative results. This was further confirmed by the failure to demonstrate IgM anti-HAV in sera from normal blood donors positive for anti-HAV and in sera with a high titer of anti-HAV collected more than 3 months after the onset of acute hepatitis type A.

With other ELISA and SPRIA tests for IgM antibody for different viral diseases, a different principle has generally been used (1, 3, 8, 21). The serum to be tested is added to an antigen-coated solid phase, followed by conjugated antihuman IgM (Fig. 3). In this system IgM and IgG antibodies compete for the antigenic sites, thus making it difficult to demonstrate IgM antibodies in the presence of larger amounts of IgG antibodies. Furthermore, it is possible for rheumatoid factor to give false-positive results for IgM antibodies in the presence of IgG antibodies (14, 20a).

In the test described here there is no competition between IgG and IgM antibodies, but only between IgM antibodies of other specificities. Furthermore, because of the selection of IgM antibodies by the anti-IgM-coated plate, the virus-antibody complex is allowed to form without interference of any possible rheumatoid factor originally present in the serum to be tested. In accordance with this we did not find any of the rheumatoid factor-containing sera to react positively in ELISA for IgM anti-HAV, although most of them contained anti-HAV when tested by competition ELISA. On the other hand, about three-quarters of the sera were found to give false-positive results when examined for rubella IgM antibodies in an ELISA test in which rubella IgM antibody was measured by attachment to rubella antigen-coated plates.

We believe that the principle described here, with binding of IgM antibodies to anti-IgM-coated plates followed by testing for the specificity of the IgM, is going to be widely used for the serodiagnosis of various infectious diseases. The test is easy to perform, it is accurate, and false-positive results due to rheumatoid factor have not been observed. During HAV infection, ELISA for IgM anti-HAV allows rapid confirmation of the diagnosis by examination of a single serum sample taken during the acute phase of illness, and appropriate measures can therefore be taken early in the course of infection.

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LITERATURE CITED


