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# Specific Immunoglobulin M Response to Hepatitis A Virus Determined by Solid-Phase Radioimmunoassay

STANLEY M. LEMON,* CARLTON D. BROWN, DOLORES S. BROOKS, THOMAS E. SIMMS, AND WILLIAM H. BANCROFT

*Department of Virus Diseases, Division of Communicable Disease and Immunology, Walter Reed Army Institute of Research, Washington, D.C. 20012*

Immunoglobulin M antibody to hepatitis A virus (IgM anti-HAV) is found in most patients with acute type A hepatitis. To determine the duration of this IgM response as well as to confirm that IgM anti-HAV is a specific marker for acute infection, we developed a solid-phase radioimmunoassay for IgM anti-HAV. This new assay is 25-fold more sensitive than a conventional blocking radioimmunoassay for anti-HAV, and interference due to rheumatoid factor was eliminated by simultaneously testing sera against virus-free control antigen. Maximum IgM anti-HAV titers (1:6,400 to ≥1:51,200) were detected during the first 30 days after the onset of illness. Although the IgM anti-HAV titer subsequently declined 64-fold over the ensuing 90 days, low-titer IgM anti-HAV (1:100 to 1:400) persisted in many sera for 90 to 150 days. Acute sera having an IgM anti-HAV titer of ≥1:25,600 possessed a significantly higher mean IgM concentration (492 mg/dl) than acute sera with an IgM anti-HAV titer of ≤1:12,800 (344 mg/dl; $P < 0.05$). IgM anti-HAV titers did not correlate with other clinical or laboratory measures of disease severity. Detection of IgM anti-HAV proved to be both a highly specific (>99%) and a sensitive (>99%) method for the diagnosis of type A hepatitis.

A distinguishing feature of acute type A hepatitis is the frequent marked elevation of serum immunoglobulin M (IgM) (13). Whether this increase in IgM represents predominantly virus-specific antibody or a nonspecific polyclonal IgM response remains unknown. Nevertheless, antibody to hepatitis A virus (anti-HAV) appearing early in the course of hepatitis A infection is predominantly IgM (4, 17). This early antibody is almost universally present at the onset of illness and may be detected by immune electron microscopy (9, 17), radioimmunoassay (20), and enzyme-linked immunoassay (18) but not immune adherence hemagglutination (19). IgG anti-HAV appears somewhat later and is detected by all these methods.

Detection of IgM anti-HAV, if specific for acute infection, could be a powerful laboratory tool for the recognition of type A hepatitis (4, 10). Currently, the laboratory diagnosis of hepatitis A infection is limited by two features of the disease. First, fecal shedding of virus often terminates soon after the onset of symptoms, making antigen detection methods impractical (21). Second, demonstration of seroconversion, except by immune adherence hemagglutination, may be impossible due to the early appearance of high-titer antibody. The mere presence of IgG anti-HAV has very limited diagnostic value because this antibody persists for life. IgM anti-HAV, however, appears to be a useful marker for recent hepatitis A infection (3, 8, 11, 16).

We describe in this report the development of a solid-phase radioimmunoassay specific for IgM anti-HAV (IgM-SPRIA) and its application to the differentiation of type A hepatitis from hepatitis due to other viruses. Furthermore, we have determined the magnitude and duration of the virus-specific IgM response and have attempted to correlate it with the changes found in total serum IgM concentrations. (This study was presented in part at the 19th Interscience Conference on Antimicrobial Agents and Chemotherapy, Boston, Mass., 1-5 October 1979.)

## MATERIALS AND METHODS

**HAV antigen.** Two susceptible colony-bred chimpanzees, held at the Laboratory for Experimental Medicine and Surgery in Primates, Tuxedo, N.Y., were infected intravenously with MS-1 strain virus. The inocula were filtrates of feces collected from infected human volunteers (2) previously shown to contain infectious HAV (6). Stool was collected daily from each chimpanzee and subsequently screened for HAV by a radioimmunoassay modification of the enzyme-linked immunoassay of Mathiesen et al. (18). HAV antigen was prepared from a single day's collection of chimpanzee stool (HAV positive) twice extracted with phosphate-buffered saline (PBS; pH 7.4) with 0.2% sodium azide. The final material, representing a 10% (wt/vol) stool suspension, was clarified by centrifuga-

tion at 12,000 × g for 30 min. Aliquots of supernatant were kept frozen at −70°C until use. Control antigen was prepared by similarly processing preinfection stool from the same animal.

**Reagent sera.** Goat antisera to human IgM (T = 3.5 [1]) was purchased from Antibodies, Inc., Davis, Calif. Reference human serum containing a known concentration of IgM was obtained from Behring Diagnostics, Somerville, N.J. Human convalescent hepatitis A serum was collected from a patient 90 days after the onset of typical type A hepatitis and had an anti-HAV titer of 1:16,000 by immune adherence hemagglutination (test kindly performed by Robert Purcell). An anti-HAV-negative serum pool was prepared by pooling sera from four healthy laboratory workers who lacked antibody to hepatitis A.

**Test sera.** Sera from patients with acute hepatitis A infection were collected during three separate epidemics. One outbreak occurred in 1977 near Anchorage, Alaska, among both military personnel and their dependent children on a large army post (M. W. Benenson, E. T. Takafuji, W. H. Bancroft, S. M. Lemon, M. C. Callahan, and D. A. Leach, Am. J. Epidemiol., in press). A second outbreak involved only soldiers stationed at Schofield Barracks, Hawaii, in 1978, and a third epidemic involved North Atlantic Treaty Organization military personnel and their dependents stationed near Brussels, Belgium, during 1979. Sera from cases of hepatitis B and non-A non-B hepatitis were collected as part of an ongoing study of U.S. army soldiers hospitalized within the United States, the Federal Republic of Germany, and the Republic of Korea. Rheumatoid factor (RF)-positive sera, collected from patients with rheumatoid arthritis, were kindly provided by Oliver Lawless of the Rheumatology Service, Walter Reed Army Medical Center, Washington, D.C.

**Preparation of [$^{125}$I]IgG.** IgG in 5 ml of human convalescent hepatitis A serum was precipitated by the sequential addition of 45 and 35% $(NH_4)_2SO_4$. The final precipitate was dialyzed extensively against 0.25 M phosphate buffer (pH 7.4) and passed through a Sephadex G200 column (107 by 1 cm; Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) equilibrated with the same buffer. The second major protein peak eluted was filter concentrated with an XM150 Diaflo filter (Amicon Corp., Lexington, Mass.) and then pressure dialyzed against 0.01 M phosphate buffer (pH 6.8). The concentrate was applied to a 10-ml diethylaminoethyl-Sephadex A25 column (Pharmacia Fine Chemicals) and eluted with the pH 6.8 buffer. The single protein peak eluted contained approximately 2 mg of IgG per ml and was frozen in fractions at −20°C until use. A 20-μg portion of IgG was iodinated with 500 μCi of $^{125}$I by a modification of the chloramine-T method (14). [$^{125}$I]IgG was separated from free $^{125}$I by passage through a 5-ml Sephadex G25 column (Pharmacia Fine Chemicals) and stabilized by the addition of 25% (vol/vol) fetal calf serum and KI (1 mg/ml). The radiolabeled IgG possessed a specific activity of approximately 10 μCi/μg of protein, and over 98% of the $^{125}$I was protein bound. Iodinated goat IgG anti-IgM was prepared in a similar fashion, with the exception that anion-exchange chromatography was deleted.

**IgM-SPRIA procedure.** The IgM-SPRIA procedure (Fig. 1) finally adopted was similar to that first proposed by Duermeyer and van der Veen (7). Ninety-six-well, flexible polyvinyl chloride microtiter U plates (Dynatech Laboratories, Alexandria, Va.) were rinsed with tap water and shaken dry. Only the central 60 wells of each plate were used. A 100-μl amount of goat anti-human IgM, diluted 1:1,000 in 50 mM sodium carbonate buffer (pH 9.0), was delivered to each well, and the plates were placed in a humidified chamber at 30°C for 4 h. The plates were then washed five times with PBS containing 0.05% Tween-20 (PBS-T), and each well was filled with 1% bovine serum albumin (Calbiochem, La Jolla, Calif.) in Dulbecco PBS (GIBCO Laboratories, Grand Island, N.Y.) with 0.2% sodium azide and 0.005% phenol red. After an overnight incubation at 4°C, the microtiter plates were washed seven times with PBS-T, and 50 μl of a test serum dilution was added to each well. Two replicate wells were used for each serum dilution-antigen combination. Sera were diluted 1:10 in PBS for screening purposes. The plates were incubated for 4 h at 30°C and then washed seven times with PBS-T. A 20-μl amount of HAV or control antigen was delivered to each well, and the plates were reincubated overnight at 4°C. The plates were washed again seven times with PBS-T, and 20 μl of the anti-HAV-negative serum pool (diluted 1:100 in PBS) was added to each well. The plates were held at room temperature for 15 min. Without further washing, 20 μl of [$^{125}$I]IgG-anti-HAV (approximately 250,000 cpm diluted in 10% fetal calf serum in PBS) was added to each well, and the plates were gently agitated and then placed at 4°C for 2.5 h. After they were washed seven times as above, the plates were tapped dry, and individual wells were cut apart. Bound $^{125}$I was measured by counting individual wells in a Nuclear-Chicago model 1185 gamma counter (Tracor Analytic, Elk Grove, Ill.).

In each microtiter plate, four serum samples were included as IgM anti-HAV-negative controls; two were positive and two were negative for IgG anti-HAV. From the eight wells filled with these four serum samples, a "negative mean" counts per minute value was calculated. Subsequently, for each test serum dilution, the mean counts per minute value of the replicate test wells was divided by the negative mean to yield a positive/negative ratio (P/N value). P/N values equal to or greater than 2.2 were considered positive (see below). Also included on each plate were standard dilutions of an IgM anti-HAV-positive control serum. The titer of IgM anti-HAV was defined as the highest serum dilution yielding a P/N of 2.2 or greater. Each serum specimen was also tested at a 1:10 dilution against HAV-negative control antigen to facilitate detection of those sera giving false-positive results due to the presence of RF. P/N values for the control antigen reactions were calculated in a similar fashion.

**Routine hepatitis serology.** Non-class-specific antibody to HAV was detected by either a radioimmunoassay modification of the enzyme-linked immunoassay of Mathiesen et al. (18) or by a commercial radioimmunoassay (HAVAB, Abbott Laboratories, North Chicago, Ill.). Hepatitis B surface antigen, antibody to HBsAg, and antibody to hepatitis B core antigen were all detected by specific commercial ra-

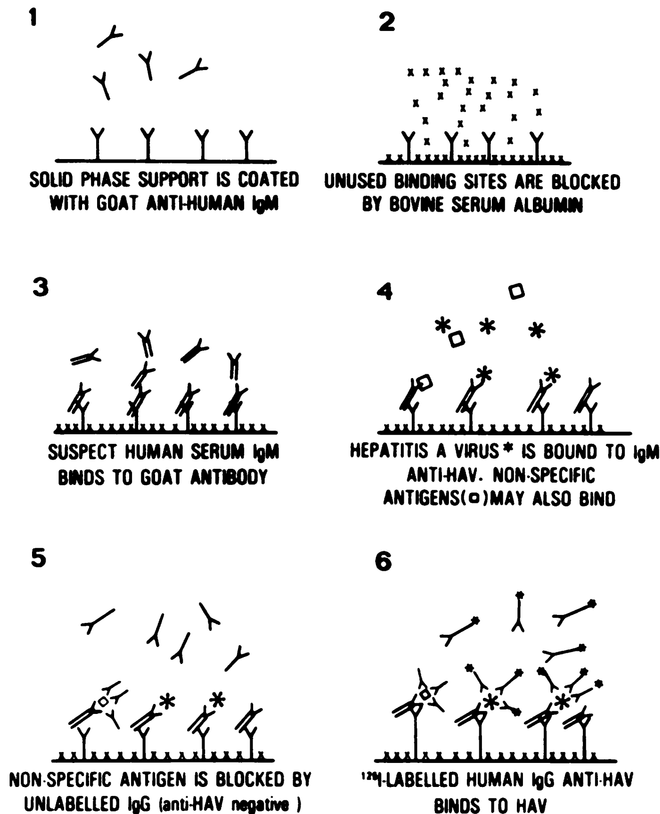


FIG. 1. *Outline of the SPRIA procedure for detection of IgM antibody to hepatitis A virus (IgM anti-HAV).*

dioimmunoassays (AUSRIA II, AUSAB, and CORAB, respectively, Abbott Laboratories, North Chicago, Ill.). Rheumatoid factor was assayed by a latex agglutination procedure (ICL Scientific, Fountain Valley, Calif.), and serum IgG and IgM concentrations were measured with radial immunodiffusion plates prepared by Behring Diagnostics.

**Separation of IgM from IgG by rate-zonal centrifugation.** A 100-μl amount of serum was layered onto a 10-ml 10 to 40% linear sucrose gradient in normal saline, placed in an SW41 rotor, and centrifuged at 35,000 × $g$ for 24 h at 4°C in a Beckman L5-50 B centrifuge (Beckman Instruments, Palo Alto, Calif.). Fractions (0.25 ml) were collected from the bottom of the gradient, and the protein content of each fraction was estimated from the optical density at 280 nm. Individual fractions were tested undiluted for anti-HAV (by HAVAB), as well as for IgG and IgM content.

## RESULTS

**Solid-phase separation of IgM and IgG.** Pilot studies optimized the binding of IgM to the solid-phase support and also established the immunoglobulin class specificity of the goat anti-human IgM. Various dilutions of goat anti-IgM (100 μl) in carbonate buffer (pH 9) were placed in replicate wells of microtiter plates, which were then incubated at 30°C for 4 h, conditions which we previously found to be optimal. After washing, plates were incubated overnight with 1% bovine serum albumin as described in Materials and Methods. A reference standard containing 10 μg of IgM per ml was then added to each well, and after 4 h at 30°C, the plates were washed and [$^{125}$I]anti-IgM was added. Bound $^{125}$I was measured after an additional 2.5 h of incubation. A 1:1,000 dilution of the goat antiserum provided optimal binding of IgM to the plate (Fig. 2A). A quantitative measure of the solid-phase adsorption of IgM was obtained by repeating this procedure with the 1:1,000 dilution of goat anti-IgM and various dilutions of the IgM reference standard (Fig. 2B). IgM was detected on the plate even when the reference standard contained as little as 30 ng of IgM per ml (700 cpm bound), and IgM binding sites appeared saturated at IgM concentrations of 30 μg/ml or greater (approximately 4,000 cpm bound). However, when the IgM standard was replaced with purified IgG anti-HAV (20 to 2,000 μg/ml), no antibody was detected (75 to 100 cpm bound) (data not shown). Thus, the goat anti-human IgM did not

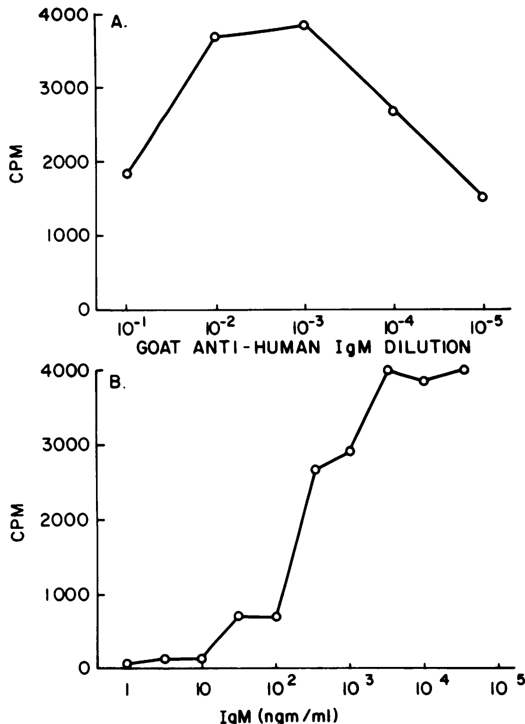


FIG. 2. *Binding of human IgM to antibody-coated microtiter plates. (A) Microtiter wells coated with various dilutions of goat anti-human IgM were incubated with a dilution of human serum containing 10 μg of IgM per ml. IgM bound to the wells was detected by [$^{125}$I]anti-IgM and was greatest when wells were coated with a 1:1,000 dilution of goat anti-IgM. (B) Wells coated with goat anti-IgM (1:1,000 dilution) were incubated with various concentrations of human IgM. IgM was bound to the microtiter plate when it was incubated with as little as 30 ng of IgM per ml. IgM-binding sites were saturated at ≥30 μg of IgM per ml.*

react with IgG, and the IgG anti-HAV preparation was free of contaminating IgM (less than 15 ng of IgM per mg of IgG).

**Antigen binding to adsorbed IgM and detection of IgM anti-HAV.** The IgM SPRIA was carried out as described in Materials and Methods by employing various dilutions of HAV antigen (1 to 10% [wt/vol] fecal extracts) with a 1:100 dilution of a serum specimen (B021) obtained from a patient 11 days after the onset of hepatitis A. P/N values were determined based on results obtained with two anti-HAV-negative sera and two anti-HAV-positive sera, all collected from asymptomatic individuals without a recent history of hepatitis. As shown in Fig. 3A, although HAV could be detected in the 1% (wt/vol) stool extract, greater P/N values were found with more concentrated antigen preparations. Altogether, P/N values greater than 2.2 were obtained with 10 of 14 chimpanzee stool extracts previously shown to contain HAV. Preinfection chimpanzee stools (control antigen) tested in a similar fashion repeatedly were negative (P/N value of 1.3 or less).

Employing a 10% fecal extract as antigen, we tested 10-fold dilutions of serum B021. IgM antibody could be detected at a serum dilution of $10^{-4}$ but not $10^{-5}$ (Fig. 3B). Additional experiments were carried out to ascertain the optimal incubation time for the $^{125}$I-anti-HAV antibody. Maximum counts per minute were bound by 2 h, and further incubation did not significantly increase the amount of $^{125}$I-labeled antibody bound.

Inasmuch as the [$^{125}$I]anti-HAV employed was not monospecific for hepatitis A virus, pooled human anti-HAV-negative sera were added to the microtiter wells before the addition of [$^{125}$I]anti-HAV. This step was included to improve test specificity if both test serum IgM and

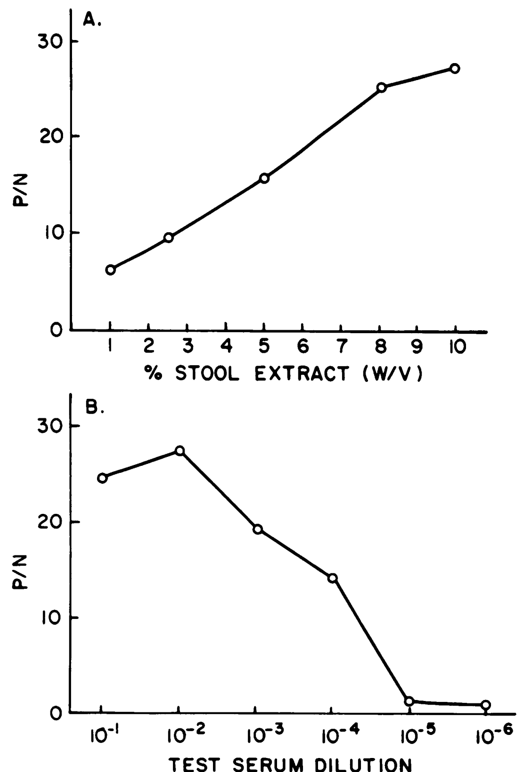


FIG. 3. *Detection of HAV and IgM anti-HAV by radioimmunoassay. (A) Antigen dilution curve obtained with an HAV-containing fecal extract tested against a serum specimen (B021) known to contain IgM anti-HAV. (B) Dilution curve for B021 test serum tested against the 10% (wt/vol) fecal extract.*

[125I]IgG anti-HAV preparations contained antibody reactive to a non-HAV component of chimpanzee fecal extract. It could also reduce interference caused by the presence of IgM RF in test serum. A 1:100 dilution of this anti-HAV-negative serum pool did not reduce the P/N values obtained with test sera containing IgM anti-HAV, but it did improve specificity with one of two HAV antigen preparations evaluated. This blocking step was employed in all subsequent testing.

To ascertain the range of P/N values obtained with normal sera, 100 anti-HAV-negative sera were tested at a 1:10 dilution. The mean P/N value was 1.12 (standard deviation, 0.359). Subsequently, sera yielding a P/N value greater than 3 standard deviations from this mean (i.e., ≥2.2) were considered to be positive in the IgM-SPRIA. An additional 22 sera collected from individuals without a recent history of hepatitis but containing anti-HAV were also tested. The mean P/N of these sera was 1.14 (standard deviation, 0.303), and the highest P/N value obtained was 1.88.

The sensitivity of the IgM-SPRIA was compared with that of the HAVAB test by determining antibody titers in nine sera collected during the first 12 days of illness (Table 1). The geometric mean titer obtained with the IgM-SPRIA was 25-fold higher than that determined by HAVAB (1:17,113 versus 1:681).

TABLE 1. *Anti-HAV titers by commercial radioimmunoassay (HAVAB) and IgM-SPRIA in acute-phase sera*

| Serum no. | Day of illness | Reciprocal anti-HAV titer[a] | |
|---|---|---|---|
| | | HAVAB | IgM-SPRIA |
| 1 | 7 | 640 | 6,400 |
| 2 | 10 | 1,280 | 6,400 |
| 3 | 9 | 2,560 | 12,800 |
| 4 | 9 | 2,560 | 51,200 |
| 5 | 12 | 320 | 12,800 |
| 6 | 12 | 1,280 | 51,200 |
| 7 | 6 | 80 | 12,800 |
| 8 | 8 | 320 | 12,800 |
| 9 | 2 | 640 | 51,200 |

[a] Geometric mean titers for anti-HAV by HAVAB and IgM-SPRIA were 681 and 17,113, respectively.

**Diagnostic sensitivity and specificity of the IgM-SPRIA.** Four groups of sera were tested at a 1:10 dilution in the IgM-SPRIA (Table 2). The first group consisted of sera collected from patients within 60 days of the onset of acute hepatitis A during three separate epidemics. Of these 110 sera, 109 yielded a P/N value >2.2. The single serum specimen (B094) which gave a negative result was collected less than 24 h after the onset of symptoms of hepatitis and was HAVAB negative as well. Nevertheless, a serum specimen collected from this individual after 3 months was anti-HAV positive and possessed an IgM-SPRIA titer of 1:400. The diagnostic sensitivity of the IgM-SPRIA for acute hepatitis A infection was therefore 109/110 or >99%. Sera from all three hepatitis A outbreaks yielded similar P/N values.

The second group consisted of 112 sera from patients with acute hepatitis B. These sera were all hepatitis B surface antigen or hepatitis B core antibody positive or both, and 77 of the 112 were anti-HAV positive (by HAVAB) as well. Only 1 of these 112 sera yielded a P/N value >2.2 in the IgM-SPRIA test. This one serum specimen (K009) was repeatedly positive, and although it also contained RF, it possessed anti-HAV (detectable by HAVAB) in IgM fractions after rate-zonal centrifugation (data not shown). Anti-HAV also remained in the IgM fraction after centrifugation in an acetate-buffered sucrose gradient (pH 4) (22), suggesting that the anti-HAV was not IgG antibody bound in immune complexes. Nevertheless, even including this one serum as a false positive, the specificity of the IgM-SPRIA (defined as the ability of the assay to distinguish acute hepatitis A from acute hepatitis B) is 111/112 or >99%. A third group of 17 sera was collected from patients with acute non-A non-B hepatitis (all IgG anti-HAV negative), and none of these sera gave an IgM anti-HAV P/N value >2.2 (specificity 100%). The fourth group of sera tested was collected from 11 patients with rheumatoid arthritis, all of whom were strongly RF positive. Three of these sera (27%), two of which were HAVAB positive, yielded P/N values greater than 2.2 but consid-

TABLE 2. *Diagnostic sensitivity and specificity of IgM-SPRIA test*

| Source of sera | No. tested | P/N <2.2 | | | P/N >2.2 | | |
|---|---|---|---|---|---|---|---|
| | | No. | P/N mean | SD | No. | P/N mean | SD |
| Acute[a] hepatitis A | 110 | 1 | 1.3 | | 109 | 14.42 | 5.0 |
| Acute hepatitis B | 112 | 111 | 1.03 | 0.32 | 1 | 15.6 | |
| Acute non-A non-B hepatitis | 17 | 17 | 1.15 | 0.29 | 0 | | |
| Rheumatoid arthritis | 11 | 8 | 1.37 | 0.27 | 3 | 2.93 | 1.02 |

[a] <60 days from onset of symptoms.

erably lower than those obtained with most acute hepatitis A sera (Table 2).

Because of the false-positive results apparently related to RF, all IgM-SPRIA-positive sera were tested in an identical fashion against a control antigen preparation (preinfection chimpanzee fecal extract). The results obtained with RF-positive sera are shown in Table 3. All of the rheumatoid arthritis sera, but none of the RF-positive hepatitis sera, resulted in a P/N value >2.2 against control antigen. It is of special interest that the acute hepatitis B serum (K009) described above also yielded a negative result when tested against the control antigen, a finding in agreement with the presence of anti-HAV in IgM fractions after rate-zonal centrifugation.

**Duration of the IgM anti-HAV response.** Paired serum specimens, collected approximately 100 days apart, provided an opportunity to study sequential changes in IgM anti-HAV (Fig. 4). Two patients with sera fortuitously collected before the onset of hepatitis, and the one patient (B094) described above with anti-HAV-negative serum collected on the first day of illness, demonstrated seroconversion in the IgM-SPRIA test. On the other hand, high P/N values returned toward normal in 16 patients who had both serum samples obtained after the onset of symptoms. Convalescent results fell within the normal range in 10. In some patients, however, IgM anti-HAV was still detectable 85 to 135 days after the onset of symptoms.

The duration of the IgM anti-HAV response was defined further by testing twofold dilutions of sera from the Belgium outbreak (Fig. 5). Maximum IgM anti-HAV titers were present during the first 30 days after the onset of illness. A 64-fold reduction in geometric mean IgM antibody titer subsequently occurred by 91 to 120 days. Many serum samples collected as late as 91 to 150 days after the onset of symptoms still contained IgM-HAV detectable at serum dilutions of 1:200 to 1:400. All of these sera were RF negative and yielded a P/N <2.2 with control antigen. To rule out the unlikely possibility that these late low-titer sera reflected the inadvertent detection of high-titer IgG antibody, immunoglobulin fractions separated by rate-zonal centrifugation were tested by HAVAB. Anti-HAV activity was present in the IgM fractions (Fig. 6).

TABLE 3. *IgM-SPRIA results obtained with RF-positive sera tested against HAV or control antigen preparations*

| Source | RF[a] | P/N value | |
|---|---|---|---|
| | | HAV antigen | Control antigen |
| Acute hepatitis A | + | 14.2 | 0.6 |
| | + | 9.7 | 1.0 |
| | + | 31.0 | 0.7 |
| | + | 28.3 | 0.6 |
| | + | 21.4 | 0.8 |
| | + | 36.1 | 0.9 |
| | + | 22.8 | 0.8 |
| Acute hepatitis B | + | 15.6 | 1.36 |
| Rheumatoid | + | 4.1 | 7.8 |
| arthritis | + | 2.2 | 7.4 |
| | + | 2.5 | 8.3 |

[a] Determined by latex slide agglutination.

**Correlation of IgM anti-HAV with serum IgM concentration and disease severity.** Total serum IgM was determined in sera collected from both symptomatic and asymptomatic individuals during the Belgium outbreak. The serum IgM fell in the normal range (50 to 280 mg/dl) in individuals with no serological evidence of recent hepatitis A but was elevated, sometimes far above normal, in 14 out of 24 patients (58%) who had serum collected during the first 35 days of illness (Fig. 7). Thus, the highest serum IgM concentrations occurred during the period when IgM anti-HAV titers were maximum. Those sera possessing exceptionally high IgM anti-HAV titers (≥1:51,200) generally had elevated total serum IgM as well. To test the hypothesis that sera with high anti-HAV titers had significantly greater serum IgM concentrations, sera collected within 35 days of onset of disease were analyzed in greater detail. The mean serum IgM concentration was 492 mg/dl in those sera having an IgM anti-HAV titer of ≥1:25,600 but only 344 mg/dl in those sera with an antibody titer of ≤1:12,800 ($P < 0.05$ by the one-sided $t$-test). There was no significant correlation between the IgM anti-HAV titer and the maximum observed serum bilirubin or serum aspartate aminotransferase. Also, no correlation was evident between the IgM anti-HAV titer and the age, sex, presence or absence of fever, diarrhea, and either myalgia or arthralgia.

## DISCUSSION

The IgM-SPRIA procedure described in this report represents an improvement over other available methods for the diagnosis of acute hepatitis A infection. We have shown that detection of IgM anti-HAV permits a reliable diagnosis to be made with testing of a single serum specimen, thus avoiding the delay inherent in testing paired serum specimens by other methods. An alternative method for the detection of IgM antibody, based upon the removal of IgG by incubation with staphylococcal protein A before testing for anti-HAV (3), may be confused in some patients by the early appearance of IgG antibody (11). We found substantial IgG anti-

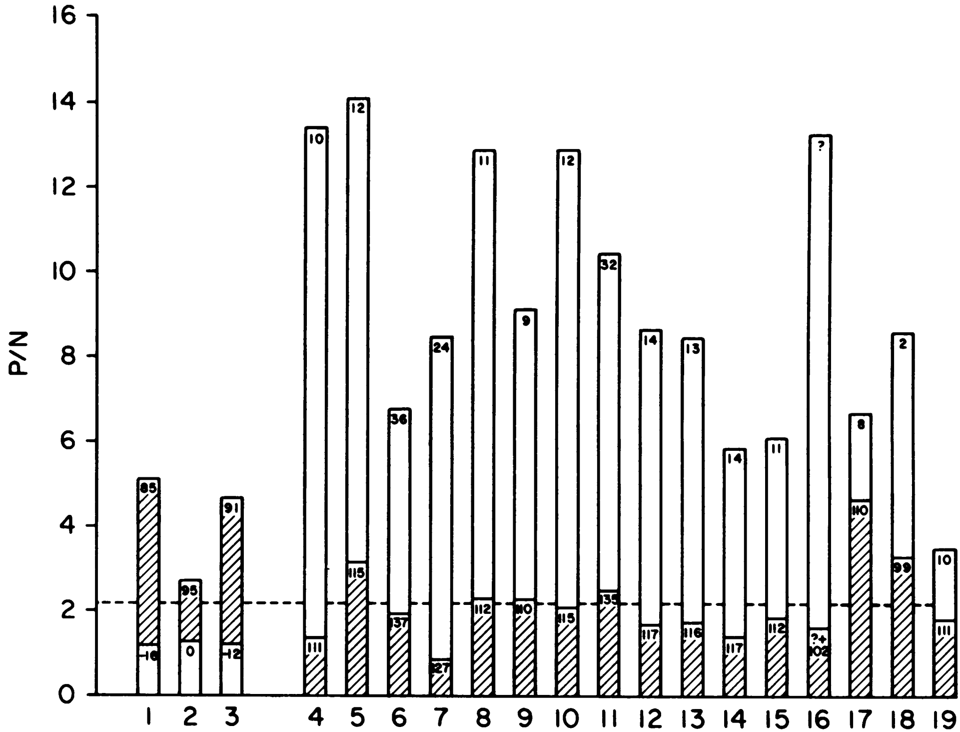


FIG. 4. *Sequential changes in IgM anti-HAV occurring in paired serum specimens from 19 patients with acute hepatitis A. Open and shaded bars represent P/N values found with the first and second serum specimens, respectively. The number at the top of each bar indicates the day of illness when that serum specimen was collected (negative sign indicates days preceding infection). Patients 1 through 3 were initially bled before the appearance of IgM anti-HAV and demonstrated seroconversion in their second serum specimen. The other 16 patients (4 through 19) were initially bled after the onset of illness and appearance of IgM anti-HAV. All sera were tested at a 1:10 dilution.*

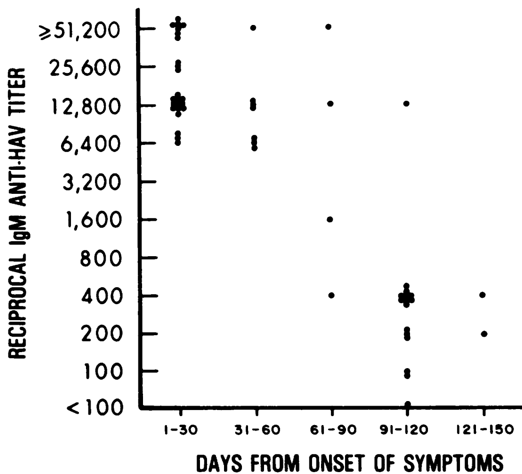


FIG. 5. *Duration of IgM antibody response as determined by IgM-SPRIA testing of 53 sera from 42 patients studied during the Belgium outbreak.*

HAV present as early as 2 days after the onset of symptoms (Fig. 6). Also, adaptation of commercial radioimmunoassays such as HAVAB to these adsorption procedures may be technically difficult and, if done improperly, misleading, as evidenced by the recent report of a "pseudo-outbreak" of hepatitis A (5).

Based upon the results of testing 239 patients with acute infection due to hepatitis A, hepatitis B, or non-A non-B viruses, we have concluded that the IgM-SPRIA assay has both extreme diagnostic sensitivity (>99%) and specificity (>99%). The single patient (B094) with recognized hepatitis A infection who did not yield a positive result was tested on the first day of illness and was unusual in that she had no demonstrable anti-HAV activity by any radioimmunoassay technique. This must be recognized as a potential pitfall in the serological diagnosis of acute hepatitis A. On the other hand, the patient with acute hepatitis B (K009) who gave a false-positive result in the IgM-SPRIA test is also of special interest. Anti-HAV was detectable in IgM fractions prepared by rate-zonal centrifugation of this rheumatoid-factor-positive serum specimen. Although this finding could reflect in vivo or in vitro binding of IgG anti-HAV to IgM rheumatoid factor, this seems unlikely because

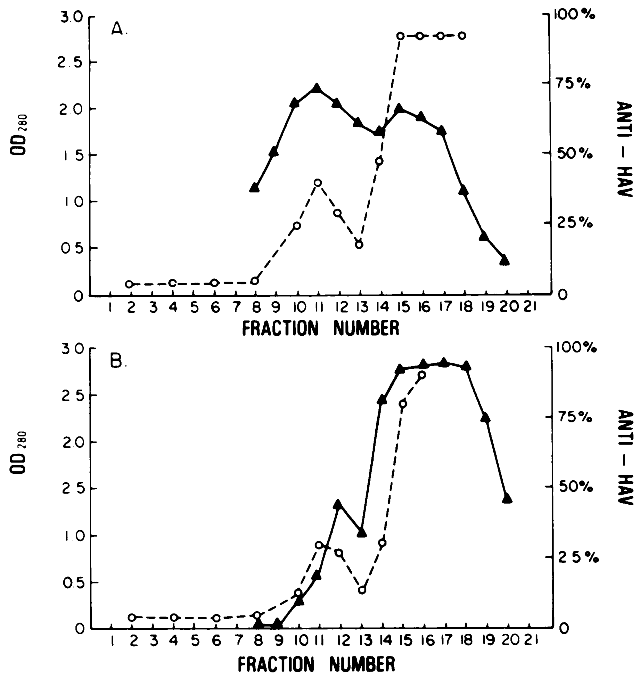


FIG. 6. *Sucrose-density gradient centrifugation of acute and convalescent hepatitis A sera. Fractions were collected from the bottom of the gradient and were tested for anti-HAV content by a commercial radioimmunoassay. IgM but not IgG was detected in fractions 10 through 12. (A) Serum collected 2 days after the onset of symptoms: IgM-SPRIA titer was ≥1:51,200. (B) Serum from the same patient collected 97 days later, IgM-SPRIA titer was 1:400. Symbols:* (▲) *percent blocking activity in the anti-HAV radioimmunoassay;* (O) *optical density at 280 nm ($OD_{280}$).*

centrifugation of the serum in acid-sucrose gradients (pH 4.0) did not shift anti-HAV activity from the IgM to the IgG zone (22). This particular patient was an American soldier stationed in the Republic of Korea. It seems likely that this positive IgM-SPRIA result represents a true recent infection with hepatitis A in a patient simultaneously infected with hepatitis B.

Compared with the commercial radioimmunoassay (HAVAB), the IgM-SPRIA is 25-fold more sensitive in the detection of IgM antibody at high dilutions of serum (Table 2). The extreme sensitivity of the newer assay is most likely due to the nature of the antibody-antigen "sandwich" constructed. Most of the HAV antigen we used was virion associated as judged by the results of CsCl equilibrium density centrifugation and molecular exclusion column chromatography (results not shown). During the radioimmunoassay procedure it is probable that intact HAV virions bind to relatively small numbers of IgM antibody molecules affixed to the solid phase. These virions would be expected to bind numerous [$^{125}$I]anti-HAV IgG molecules. Thus, many radiolabeled IgG antibody molecules would be bound as an end result of the presence of relatively few IgM anti-HAV molecules (Fig. 1). A second advantage inherent in the design of this assay is that IgM is separated from IgG antibody before the addition of antigen. This obviates the possibility of IgG competing with IgM for binding sites on the antigen and is probably at least partially responsible for the ability of the assay to detect low-titer IgM antibody even several months after the acute infection. This general method is clearly suited for use with many other virus systems and has been used successfully by others for the detection of IgM antibody to rubella virus, and the core component of hepatitis B virus as well as HAV (8, 12, 15).

A potential disadvantage to the assay as described is that IgM RF may occasionally lead to a false-positive result. This occurred with 3 of 11 RF-positive sera from patients with rheumatoid arthritis. RF interference appears to be due in part to RF binding radiolabeled IgG and not to RF binding IgG anti-HAV present in the serum, since one of these false-positive sera was anti-HAV negative. The frequency of false-positive tests due to RF observed by us was less than that found by Duermeyer and van der Veen (8) using radiolabeled whole IgG in an assay similar to our IgM-SPRIA test. RF interference

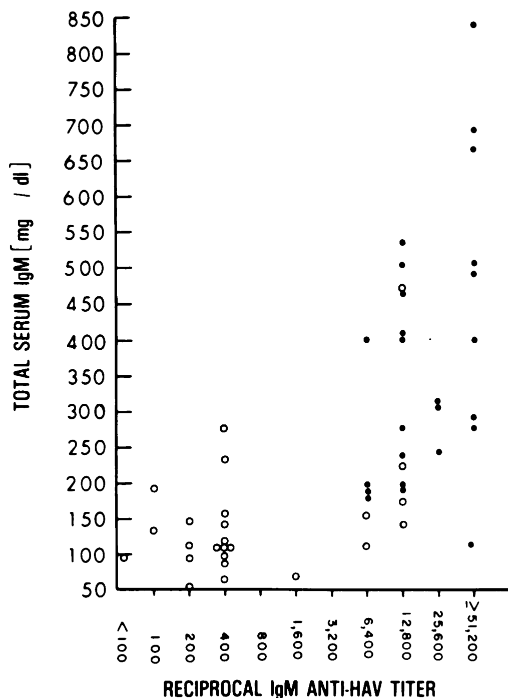


FIG. 7. *Relationship of serum IgM concentration to IgM anti-HAV titer in 50 sera collected during the Belgium outbreak.* ●, *Sera collected within 35 days of onset of illness;* ○, *sera collected between 36 and 139 days.*

may have been reduced in our assay by the inclusion of a blocking step with pooled human anti-HAV-negative serum (Fig. 1, step 5). False positives could be eliminated by substituting $^{125}$I-radiolabeled F(ab')$_2$ fragments for whole IgG anti-HAV (8). An alternative approach would be that which we employed, testing each serum specimen against an HAV-negative control antigen. This simple method allowed us to detect false-positive reactions without sacrificing sensitivity (Table 3). A recent report published while this manuscript was in preparation (10) describes a similar assay for IgM anti-HAV. It is most surprising that these authors did not find false-positive results when RF-positive sera were tested.

IgM anti-HAV was detectable in most sera even 90 to 120 days after the onset of illness (Fig. 5). Earlier studies suggesting that IgM antibody persists for only 28 to 80 days (3, 11) may be readily explained by the greater sensitivity of our method for detecting this antibody. It is of interest that the period of high-titer IgM antibody coincided generally with the period of elevated total serum IgM. Serum IgM concentration appeared to be related to IgM anti-HAV titer when all the sera collected during the Belgium outbreak were examined (Fig. 7). Because this relationship might simply reflect the acute-phase nature of both IgM responses, we analyzed in greater detail results obtained with acute sera only. Acute sera with high IgM anti-HAV titers had a significantly higher mean IgM concentration than acute sera with lower anti-HAV titers. Therefore, it seems likely that although some of the increase in serum IgM may be composed of antibody directed to antigens other than the HAV virion, a substantial fraction of this IgM is probably virus specific. However, direct determination of the percentage of IgM reacting with the virus will be required to confirm this point.

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