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# Increase in Immunoglobulin M Antibodies Against Gut Bacteria During Acute Hepatitis A

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The marked increase in the total serum immunoglobulin M (IgM) is a characteristic feature of acute hepatitis A. To study the nature of this IgM, we assayed serial titers of IgM antibodies against various antigens during and after acute hepatitis A. The antibodies against blood group antigen remained unchanged throughout the observation period. Thus, the production or metabolism of IgM was not nonspecifically altered. The IgM antibody against hepatitis A antigen decreased and finally disappeared during convalescence as expected. However, its time course did not correlate quantitatively with the concentration of the total serum IgM. In contrast, IgM antibodies against gut bacteria *Bacteroides fragilis* and *Streptococcus faecalis* were considerably elevated in all patients at the onset of the disease, and they normalized similarly to the total IgM during convalescence. IgM antibodies against *Escherichia coli* were elevated only in some of the patients. The data suggest that the amount of IgM antibodies against gut bacteria contributes significantly to the increase in the total serum IgM in acute hepatitis A.

Elevated levels of immunoglobulin are common in many different liver diseases (4, 9, 11). Probably the most drastic effect is the marked increase in the total serum immunoglobulin M (IgM) during the acute phase of hepatitis A, the nature of which is essentially obscure. We tested three different hypotheses to explain the elevated level of IgM: (i) the production or metabolism of IgM could be altered in toto; (ii) the increase in IgM could be caused by the high titers of IgM antibodies against hepatitis A virus (IgM anti-HAV); (iii) other antigens, not related to HAV, could induce high titers of IgM antibodies during the acute disease. The results of this study rule out the first two hypotheses and support the third one: very high titers of IgM antibodies against certain gut bacteria suggest that bacterial antigens could make up the marked increase in the serum IgM during acute hepatitis A.

## MATERIALS AND METHODS

**Patients.** The sera of patients with acute hepatitis A were collected at the time of admission to the hospital ($T_0$) and at 6 weeks ($T_1$), 3, 6, and 12 months later. In each case, a hepatitis B was serologically excluded (negative for hepatitis B surface antigen and IgM anti-hepatitis B core). An acute hepatitis A infection was diagnosed by the detection of IgM anti-HAV, which decreased during convalescence. The acute stage of the disease was verified in each case by elevated levels of serum alanine lactate aminotransferase and serum bilirubin and by liver biopsy.

**Determination of IgM anti-HAV.** Microtiter plates (Nunc flat-bottom polystyrene plates) were incubated with 5 μg of rabbit IgG fractions per ml against human IgM (μ-chain specific, absorbed with negative anti-HAV IgG; our own reagents) in 0.02 M sodium phosphate buffer (pH 7.2) for 48 h at 4°C, washed, and dried. Test sera were incubated 1:100 in 0.02 M sodium phosphate (pH 7.2)–0.13 M NaCl containing 0.1% heat-aggregated, negative anti-HAV human IgG to block the nonspecific binding of rheumatoid factor (3). After 2 h at 37°C, serum dilutions were removed by aspiration, and the wells were washed three times with washing liquid (0.5% Tween 20 in 0.2 M phosphate [pH 7.2]–0.13 M NaCl). HAV antigen was extracted from stools by shaking with 5 volumes of phosphate-buffered saline (PBS) and glass beads and clarifying for 30 min at 10,000 rpm in an SW40 rotor.

A suitable dilution of the extract was incubated for 10 h at 37°C in the microtiter wells, and subsequently 50 μl of anti-HAV horseradish peroxidase conjugate was added for 2 h at 37°C. The IgG for conjugation was isolated from a hepatitis A convalescent serum by ion-exchange chromatography. The conjugate was diluted in a pool of 30 to 50 negative anti-HAV human sera to 2 to 5 ng of conjugate per ml. After three washings, *ortho*-phenylenediamine and $H_2O_2$ (3) were added, and after 30 min the reaction was stopped by 2 M $H_2SO_4$. The reaction product was measured at 493 nm in a photometer.

Previous studies had shown that in this assay the color was proportional to the ratio between anti-viral IgM and the total IgM up to serum dilutions of 1:5,000 (3). For standardization of the assay, a highly positive reference serum was diluted 1:100 in buffer (corresponding to 1,000 arbitrary units of IgM anti-HAV) and thereafter to 1:400, 1:1,600, 1:6,400, 1:25,600, and

1:120,400 (corresponding to 0.98 U) in 1% negative human serum. This serum was assayed in parallel with each test run, and a calibration curve between the absorbance at 493 nm ($A_{493}$) and the units was established. With this curve, the absorbance of each positive sample was converted to units of IgM anti-HAV. Before the calculation of the $T_0/T_1$ ratio, the found values at $T_0$ or $T_1$ were multiplied by the IgM concentration of the test sera. Otherwise, the IgM anti-HAV would be considerably underestimated during the acute phase. The detection limit was 1 to 2 U, if the twofold absorbance of negative controls was taken as the cut-off point.

**Total IgM and IgG.** The total IgM and IgG were assayed by nephelometry with a commercial test system (Beckman Instruments, Inc.).

**Rheumatoid factor.** Rheumatoid factor was assayed with a latex test (Behringwerke) or a 10-fold more sensitive enzyme immune assay (Legler and Thomssen, unpublished data).

**Titration of isohemagglutinins.** Twenty microliters of serial dilutions of serum in Veronal buffer was mixed with 20 μl of a 1.5% erythrocyte suspension in U-shaped microtiter plates. To each well 40 μl of Veronal buffer (pH 7.2) was added. After 45 min at 37°C, hemagglutination was read visually. All assays were done in duplicate. The IgM nature of the isohemagglutinins was determined in some test sera by sedimentation analysis. The IgM nature was further determined by the elimination of the activity after treatment with 0.1 M mercaptoethanol and after dialysis.

**Bacterial antigens.** *Escherichia coli* O18:K1:H7, *Streptococcus faecalis* NCTC 775, and *Staphylococcus epidermidis* (strain of our laboratory) were grown on blood agar plates for 24 h at 37°C. *Bacteroides fragilis* subsp. thetaiotaomicron was grown on blood agar for 48 h at 37°C in an $H_2$-$CO_2$ atmosphere. The bacteria were suspended in PBS-EDTA (0.01 M phosphate [pH 7.4], 0.13 M NaCl, 0.0075 M EDTA) and adjusted to an $A_{620}$ (1 cm) of 3.0 or 2.0 for *B. fragilis*.

**ELISA for antibacterial IgM.** Dilutions of the sera in PBS-EDTA (200 μl) were mixed with 100 μl of bacterial suspension (for *S. faecalis*, 300 μl) in 1.5-ml V-vials. After 60 min at 37°C, the bacteria-antibody complexes were centrifuged and washed three times with 1 ml of PBS-EDTA by centrifugation. The complexes were incubated with anti-human IgM (μ-chains) globulin from rabbits which was labeled with horseradish peroxidase (DAKO) for 60 min at 37°C. The probe was diluted 1:250 in 1% bovine serum albumin. After three washings with PBS, the suspended pellets were transferred to 200 μl of the peroxidase substrate (0.04% *ortho*-phenylenediamine–0.006% $H_2O_2$ in 0.1 M sodium phosphate-citrate buffer, pH 5.0). After 30 min at ambient temperature, the enzyme reaction was stopped by the addition of 200 μl of 2 M sulfuric acid. Bacteria were spun off, and the $A_{493}$ of the supernatant was assayed.

The time course of the total IgM and of the specific IgM titers was expressed as the ratio of an acute-phase serum ($T_0$) to a convalescent serum ($T_1$). The correlation coefficients were calculated by double-logarithmic regression.

## RESULTS

**Titers of isohemagglutinins.** Isohemagglutinins are, in general, IgM antibodies. If the production or the metabolism or both of IgM were altered nonspecifically and independently of an antigenic stimulus, these antibodies ought to be increased during the acute stage of hepatitis A and decreased during convalescence, in parallel with the concentration of the total IgM.

For testing this hypothesis, we titrated the antibodies against blood group antigen B (anti-B) in paired sera from 20 patients with blood group A or O who suffered from hepatitis A. The ratio was formed between the titer of the first available serum sample ($T_0$) and a second serum sample taken 3 to 9 weeks ($T_1$) later. Anti-B remained essentially constant in spite of a clear decrease in the total IgM in all patients (Table 1).

The accuracy of the anti-B titration was sufficient to detect reliably that the titers differed by a factor of 1.5.

**Kinetics of anti-HAV.** If the increase in the total IgM were mainly due to specific IgM anti-HAV, a quantitative correlation between the two variables would be expected. However, the $T_0/T_1$ ratios of total IgM concentrations did not correlate with the ratios of IgM anti-HAV. Figure 1 shows the time course of both variables for two patients. Patient A was more typical in that both the total and IgM anti-HAV decreased from $T_0$ to $T_1$. However, the $T_0/T_1$ ratio was 11.1 for the total IgM and 2.1 for IgM anti-HAV. The accuracy of both assays excludes the possibility that the lack of correlation was caused by technical variations; only differences smaller than a factor of 1.5 could be assigned to technical errors. Patient B of Fig. 1 even showed an opposite time course of both variables: IgM anti-

TABLE 1. Lack of correlation between isohemagglutinin titers and those for the total IgM during acute hepatitis A

| $T_0/T_1$ ratio for total serum IgM | $T_0/T_1$ ratio for isohemagglutinin |
|---|---|
| 10.7 | 0.25 |
| 9.6 | 1.4 |
| 4.7 | 0.5 |
| 4.5 | 1 |
| 4 | 1 |
| 3.8 | 1 |
| 3.7 | 1 |
| 3.4 | 0.7 |
| 3.4 | 1 |
| 3 | 0.7 |
| 2.9 | 1 |
| 2.6 | 1 |
| 2.6 | 1 |
| 2.6 | 1 |
| 2.3 | 1 |
| 2 | 1 |
| 2 | 1 |
| 2 | 1 |
| 1.7 | 1 |

HAV was higher at $T_1$ by a factor of 2.5, whereas the total IgM decreased by a factor of 2.2.

Figure 2 shows the lack of correlation between the ratios of the two variables for 24 patients. The correlation coefficient was 0.07. Seventeen cases showed the most typical behavior: both parameters decreased between $T_0$ and $T_1$; thus, both $T_0/T_1$ ratios were larger than 1 (Fig. 2, right upper field). However, even for these cases, a quantitative correlation is clearly absent. In five patients, the total IgM decreased as usual, but IgM anti-HAV increased (Fig. 2, right lower field). Two patients showed the uncommon pattern that the total IgM increased during early convalescence (Fig. 2, left upper field). Again, the titer of IgM anti-HAV was independent of the total IgM and decreased in both cases.

**Kinetics of antibacterial IgM.** The titers of antibacterial IgM were determined by a newly developed enzyme immunoassay using whole bacteria as the antigen and peroxidase-labeled anti-IgM as a second reagent.

The separation of IgM from IgG by sucrose gradient centrifugation confirmed that the assay detected only IgM antibodies. A typical example of the titration curves of a serum pair ($T_0$ and $T_1$) against three different species of gut bacteria is shown in Fig. 3. The $T_0/T_1$ ratios of the titers

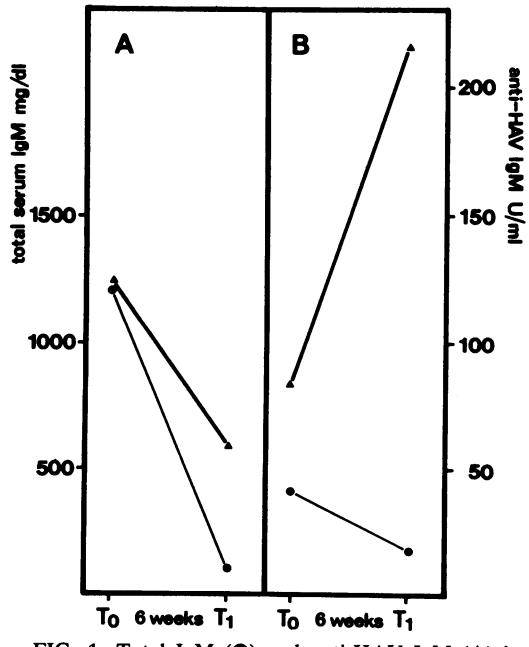


FIG. 1. Total IgM (●) and anti-HAV IgM (▲) in two selected patients with acute hepatitis A. $T_1$ was 6 weeks after the onset ($T_0$) of clinical symptoms.

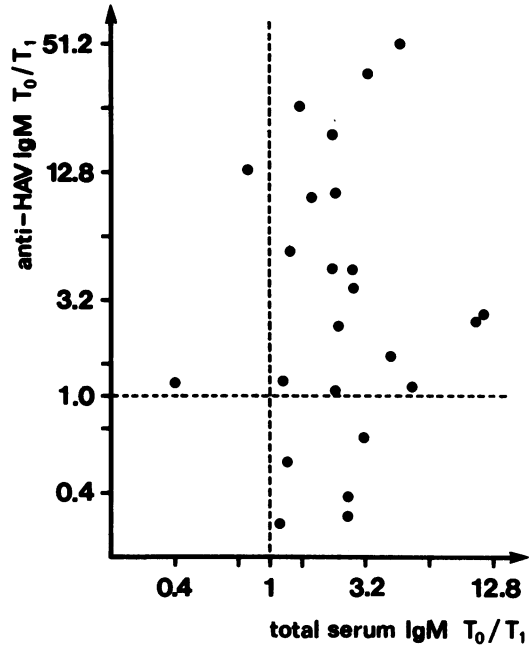


FIG. 2. Lack of correlation between the $T_0/T_1$ ratios of total IgM and those of anti-HAV IgM in 24 patients.

were read at the parallel parts of the titration curves. In the case shown in Fig. 3, the $T_0/T_1$ ratio of the total serum IgM was 3.4, and that of IgM antibodies against *B. fragilis* was 14; against *S. faecalis* the ratio was 2.1, whereas the titers against *E. coli* remained constant.

Table 2 shows the titer ratios for the IgM antibodies against these three bacteria in 18 patients.

In 17 of 18 patients, the titers against *B. fragilis* decreased from $T_0$ to $T_1$ by factors ranging between 22.6 and 2. The one patient with the constant titer also had a constant level of total IgM. The titers against *S. faecalis* decreased in 12 of 13 patients by factors ranging between 11.3 and 1.5. The behavior of the IgM antibodies against *E. coli* was inconsistent: in 5 of 11 patients, they decreased; 4 patients had constant titers, and in 2 patients the antibodies increased.

The titer ratios ($T_0/T_1$) of the antibodies against *B. fragilis* were qualitatively correlated with the ratio of the total IgM. Whenever the total IgM decreased significantly, the antibacterial IgM also decreased. In general, the specific IgM against *B. fragilis* decreased considerably more than did the total IgM during convalescence; therefore, only a weak (but significant) correlation was obtained ($r = 0.74$; $P < 0.001$). It is highly improbable that the observed titer

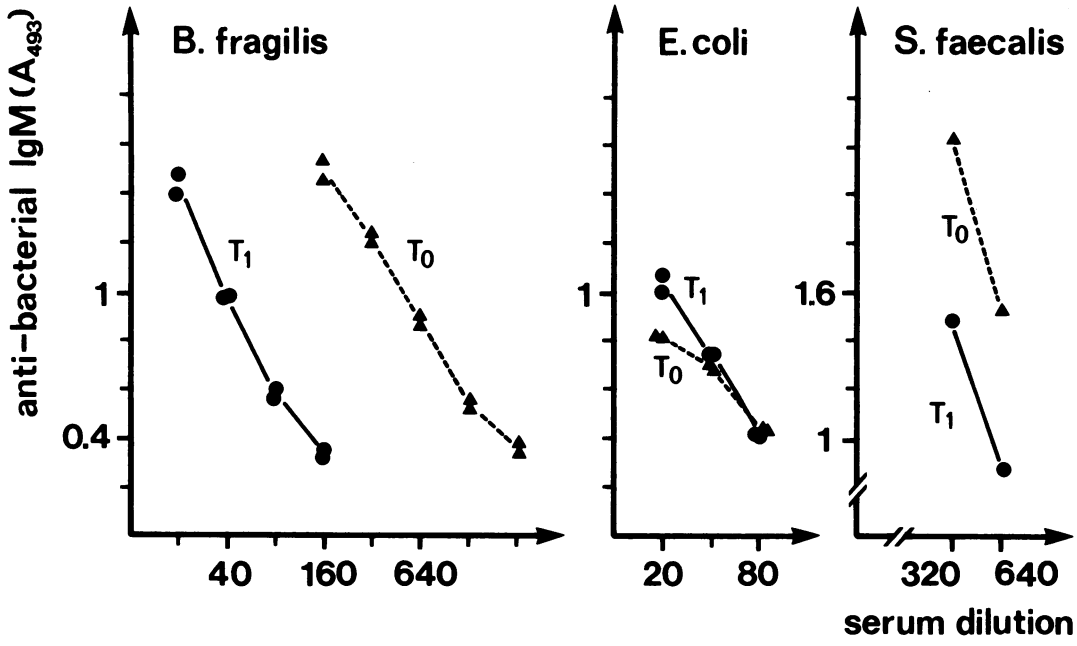


FIG. 3. ELISA of antibacterial IgM against three gut bacteria in acute-phase serum ($T_0$; ▲) and in convalescent serum ($T_1$; ●) from a hepatitis A patient. The total IgM decreased in the 6 weeks between $T_0$ and $T_1$ by a factor of 3.4.

decreases were accidental variations. In serial samples taken more than 9 weeks after $T_0$, the IgM titers against *B. fragilis* and *S. faecalis* and the total serum IgM were constant within a factor of 2. As an example, the time course of the total IgM and antistreptococcal IgM is shown for one patient during a period of 2 years (Fig. 4).

**Specificity of increased antibacterial IgM.** The increase in antibacterial IgM in hepatitis A patients is probably restricted to the antigens of gut bacteria. IgM antibodies against *S. epidermidis* were not detectable in any of the 15 patients examined.

The preabsorption of the sera with a highly concentrated bacterial suspension reduced the homologous IgM titers by factors of 10 to 100. When the concentrations of the total serum IgM were measured before and after absorption with suspensions of *B. fragilis*, *S. faecalis*, or *E. coli*, slight reductions of up to 10% were observed. However, these decreases in the total IgM were not significantly larger than the error of the method.

The IgM specificity of the enzyme immunoassay used in this study was confirmed in several samples by the sedimentation behavior of the antibacterial antibodies as 19S molecules.

Rheumatoid factor could potentially cause false-positive results. However, none of the sera was positive in a latex test at a dilution of 1:20 or in an enzyme-linked immunosorbent assay (ELISA) which was 10 times more sensitive than the latex test.

**Antibacterial IgM in hepatitis B.** Moderate increases in the total IgM occur during the acute phase of hepatitis B (8). In eight patients the titers of IgM against *B. fragilis* were assayed (Table 3). In five of six patients with clearly elevated IgM concentrations, the antibacterial titer was significantly higher at $T_0$ than at $T_1$. Two patients showed constant titers of both the total serum IgM and antibacterial IgM. In two other patients (marked in Table 3 with a asterisk), sera from the time before onset were available. In these pre-sera, the total IgM concentrations and anti-*Bacteroides* sp. IgM titers were the same as in the sera taken during convalescence (Fig. 5).

## DISCUSSION

The drastic increase in the total serum IgM in acute hepatitis A begins 3 to 4 days after the first rise in transaminases and reaches peak values at the onset of clinical symptoms. Thereafter, the IgM concentration returns to its initial values within several weeks (4).

It has been suggested that the production of immunoglobulins is nonspecifically increased during liver diseases (5). If that hypothesis were true for IgM during acute hepatitis A, IgM antibodies against all kinds of antigens would be

TABLE 2. Time course of total IgM and antibacterial IgM during acute hepatitis A

| $T_0/T_1$ ratio for total serum IgM | $T_0/T_1$ ratio for antibacterial IgM | | |
|---|---|---|---|
| | *B. fragilis* ($n$ = 18) | *S. faecalis* ($n$ = 13) | *E. coli* ($n$ = 11) |
| 9.6 | 16 | 5.7 | 4.6 |
| 8.2 | 16 | 3.5 | 2.5 |
| 4.7 | 4.6 | 1 | 0.8 |
| 4.5 | 22.6 | 3.5 | 1 |
| 4 | 11.3 | 11.3 | 2.1 |
| 3.8 | 7 | 2 | 0.6 |
| 3.7 | 11.3 | 8 | 1.6 |
| 3.4 | 14 | 2.1 | 1 |
| 3 | 7 | 4 | 4.6 |
| 2.6 | 22.6 | 2.8 | 1.2 |
| 2.6 | 4.9 | 2.8 | 0.4 |
| 2.9 | 4 | 3.5 | |
| 2.3 | 4.9 | 1.5 | |
| 2 | 4.9 | | |
| 1.9 | 6.1 | | |
| 1.7 | 2 | | |
| 1.5 | 3.2 | | |
| 1.1 | 1 | | |
| Median 2.95 | 5.5 | 3.5 | 1.1 |
| $r^a$ | 0.74 | 0.25 | 0.50 |
| $P$ | <0.001 | >0.05 | >0.05 |

[a] Correlation between the log ratio of antibacterial IgM and that of total IgM.

increased at the onset of the disease and would return uniformly to lower levels or zero during convalescence. The constancy of the isohemagglutinin during and after the disease is not consistent with a general overproduction or a decreased metabolism of IgM. This finding is in agreement with the report of Dietz et al. (2), who did not find significant differences in antibody titers against blood group antigens or Salmonella O and H antibodies when sera from acute hepatitis A patients were compared with those of normal persons.

TABLE 3. Time course of total IgM and anti-*B. fragilis* IgM in acute hepatitis B

| $T_0/T_1$ ratio for total serum IgM | $T_0/T_1$ ratio for anti-*B. fragilis* IgM |
|---|---|
| 3.3 | 2 |
| 2.2[a] | 4 |
| 2.1 | 5.7 |
| 2 | 2.5 |
| 1.4[a] | 4 |
| 1.4 | 1 |
| 1 | 1 |
| 1 | 1 |

[a] Pre-sera were available (Fig. 5).

In view of the high IgM titers against HAV at the onset, it has been suggested that a substantial part, at least, of the total IgM is directed against HAV (2, 7). A quantitative comparison of the total IgM with the antiviral IgM, however, demonstrated a clear lack of correlation between these two parameters. In fact, in a significant number of patients, contradictory kinetics were observed. Thus, the production of antiviral IgM cannot explain the drastic increase in the total IgM. A direct confirmation of this conclusion by absorption was not made because large amounts of highly purified HAV antigen, necessary for this experiment, were not available.

A satisfactory explanation for the increase in IgM at the onset of hepatitis A has not yet been found. However, the behavior of the IgM titers

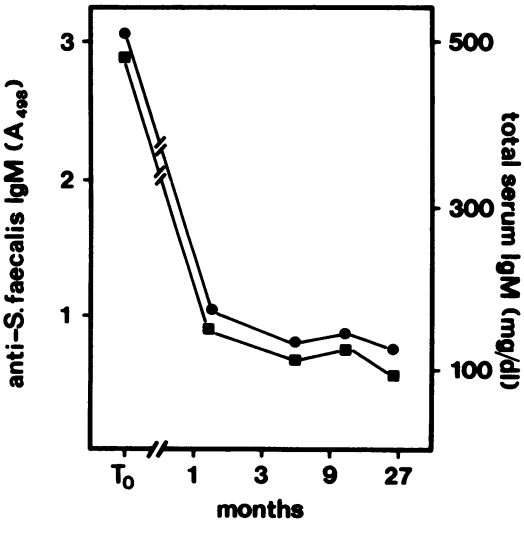


FIG. 4. Time course of total IgM (■) and anti-*S. faecalis* IgM (●) during a long observation period. The ELISA of the antibacterial IgM was done at a serum dilution of 1:640.

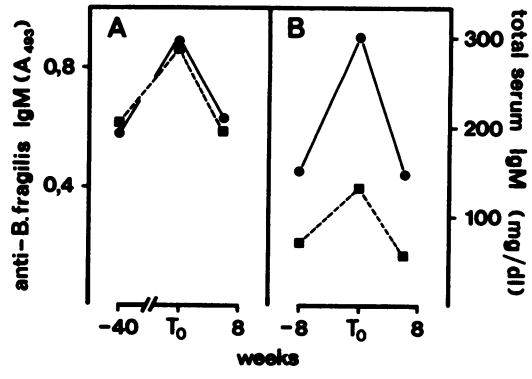


FIG. 5. Anti-*B. fragilis* IgM (●) and total IgM (■) before, during ($T_0$), and after acute hepatitis B in two patients (A and B). The serum dilution for the ELISA was 1:40.

against *B. fragilis*, *S. faecalis* and, to a lesser degree, against *E. coli* suggests that the excess of IgM is probably induced by certain antigens not directly related to HAV. The weak, but highly significant, correlation between IgM titers against *B. fragilis* and the total IgM concentration points to an important role for bacterial antigens. Absorption experiments failed to prove the antibacterial nature of the excess IgM, although a small effect was found. In addition, we used only one strain of *B. fragilis*, *S. faecalis*, or *E. coli* as the antigen. If there are great antigenic differences between the bacterial strains from different patients, only the small fraction of cross-reacting antibodies would be detectable in this study. For future work, autologous strains of the patients could be used as the antigens to investigate the nature of the increased IgM in more detail.

Elevated titers of serum agglutinins to *E. coli* and *B. fragilis* during acute hepatitis have also been reported by Triger and Wright (10). In serial sera, they found, in contrast to this study, that the titers peaked against *E. coli* 2 months after the onset of the disease. However, neither the etiology of the hepatitis nor the antibody class was clearly defined in that study.

*B. fragilis* is, together with other *Bacteroides* species, the most abundant bacterium in the gut. The finding that the titer of anti-*B. fragilis* IgM correlated best with the total IgM is consistent with the conclusion that bacterial antigens may induce a very strong IgM response during acute hepatitis A. Recently, it has been reported (6) that Kupffer cells are almost exclusively responsible for the clearance of bacterial products, such as endotoxin. It has earlier been suggested (1) that the liver has an important role as a barrier against the entrance of bacterial antigens into the bloodstream via the portal vein. Possibly, the filter effect of the reticuloendothelial system in the liver is severely impaired at the onset of acute hepatitis A. A similar effect, although less pronounced seem to occur in hepatitis B also. Further studies will be necessary to elucidate the potential role of gut bacteria in acute hepatitis.

## ACKNOWLEDGMENTS

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