Immunoglobulin M Responses to the Norwalk Virus of Gastroenteritis

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Eighty-seven serum specimens from 20 human subjects experimentally inoculated one or more times with Norwalk virus were quantitatively examined for virus-specific immunoglobulin M (IgM). A sensitive and specific radioimmunoassay for anti-Norwalk virus blocking activity was applied to whole serum and to separate IgM and IgG fractions obtained by sucrose density gradient ultracentrifugation. The peak IgM response occurred at about 2 weeks after illness, but IgM was detectable at lower titers for up to 21 weeks after infection. The IgM response was seen in volunteers who became ill, whether or not prechallenge total serum antibody was present. On long-term (27 to 42 months) rechallenge, volunteers who were previously ill and had produced IgM antibody again developed illness, and a secondary IgM response greater than the first was detected. Inoculated volunteers who did not develop illness, as well as previously ill volunteers on short-term rechallenge (4 to 14 weeks), usually failed to generate an IgM response, whether or not an IgG response had occurred. In ill subjects, the rise in IgM and IgG occurred concomitantly. Virus-specific IgM is not necessarily indicative of primary infection with Norwalk agent inasmuch as reinfec tion produces an enhancement of the IgM response. Furthermore, Norwalk-specific IgM responses do not appear to be associated with subclinical illness.

Norwalk virus is responsible for about one-third of the epidemics of viral gastroenteritis in the United States. Worldwide, 50 to 75% of adults possess serum antibody to Norwalk virus. The agent is a 27-nm virus of uncertain classification which is noncultivable in cell cultures or laboratory animals (1, 4). Norwalk illness is studied experimentally only by the oral administration to human volunteers of a bacteria-free and toxin-free infectious stool filtrate. An unusual pattern of immunity is seen in these volunteers (2, 10). Only about half of the subjects inoculated with Norwalk virus develop gastroenteritis. Although serum antibody levels rise after Norwalk illness, these responses appear to reflect infection in susceptible persons and not to have a protective role, because illness commonly occurs in the presence of serum antibody. In contrast, volunteers who resist illness usually have low to absent serum antibody levels before and after exposure to the virus. Findings similar to those found with serum have been noted with antibody levels in duodenal fluids (2, 4). Paradoxically, then, the presence of antibody to the virus and the ability to generate it constitute risk factors for this illness. When volunteers were inoculated with Norwalk virus and then rechallenged 27 to 42 months later, precisely the same volunteers who became ill on the initial challenge again became ill on rechallenge. Those who were clinically well on the first challenge remained unaffected on the second. However, short-term resistance to Norwalk illness has been noted in that most previously ill volunteers who were rechallenged 4 to 14 weeks later remained well (1, 10).

This report is a study of the presence, characteristics, and potential diagnostic and immunopathological significance of the immunoglobulin M (IgM) antibody response to Norwalk virus. Heretofore, only total serum antibody responses to Norwalk virus have been examined quantitatively by a solid-phase radioimmunoassay (RIA) blocking test (2, 5). This RIA procedure relies of necessity on carefully selected human clinical materials for its critical reagents because it has not been possible to purify Norwalk antigen from stools sufficiently to permit the preparation of useful hyperimmune animal serum. To develop an IgM antibody test specific for Norwalk virus, we found it necessary to test fractionated serum in the Norwalk RIA blocking test rather than to revise the RIA test to use an anti-human IgM reagent in a direct test. We were not able to use the more practical direct assay, probably because of procedural limitations imposed by the relatively low titer of Norwalk antigen shed in stool specimens.
MATERIALS AND METHODS

Serum specimens. Eighty-seven serum specimens from 20 previously studied human volunteers (2, 10, 12, 16), who were challenged with Norwalk virus one or more times, were analyzed in this study.

Serum fractionation. IgM was purified by sucrose density gradient fractionation of whole serum by using a modification of the procedure of Palmer et al. (9). A continuous 10 to 40% sucrose (enzyme grade; Becton, Dickinson & Co., Orangeburg, N.Y.) gradient in phosphate-buffered saline (pH 7.3) with 0.1% sodium azide was prepared in a 5-ml ultracentrifuge tube. A 0.5-ml amount of a 1:4 dilution in Veronal buffer (pH 7.3) of the serum to be fractionated was layered on top of the gradient. The sample was ultracentrifuged in a Beckman L5-75B centrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) in an SW50.1 rotor at 100,000 × g for 16 h at 4°C. Fractions (0.3 ml) were collected from the bottom of the tube, and each fraction was tested by radial immunodiffusion (Hyland Diagnostics, Deerfield, Ill.) for the presence of IgM, IgG, and IgA. Those fractions which contained IgM without trace amounts of IgG or IgA were pooled. The IgM concentration of pooled fractions was also determined by radial immunodiffusion. Pooled IgG fractions lacking IgM were analyzed similarly.

Reduction and alkylation of serum fractions. Several pooled IgM and IgG-containing serum fractions were tested for sensitivity to the reducing agent dithiothreitol. Reduction was accomplished by a modification of the procedure of Schroenloher (13). To 90 μl of the pooled serum fraction, 10 μl of 0.1 M dithiothreitol was added, and the mixture was incubated for 1 h at 37°C, followed by removal of the reducing agent by chromatography on a Sephadex G-100 column (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The protein-containing fractions were pooled, and 40 μl of a 0.5 M iodoacetamide solution per ml was added immediately to prevent reassociation of the reduced IgM.

RIA blocking test. Antibody to Norwalk virus was quantitatively measured by the previously reported RIA blocking test (2). Briefly, in this procedure microtiter plates are first coated with a high-titer anti-Norwalk human serum. A human stool specimen rich in Norwalk antigen is added, followed by the addition of dilutions of serum or serum fractions to be tested. Finally, 125I-labeled anti-Norwalk human immunoglobulin is added to the microwell. A given dilution of a test specimen is considered to be positive if it reduces the number of counts bound to the solid phase by at least 50% compared with the buffer control. Repeat testing of sera and fractions in RIA has yielded reproducible results. When reduced serum fractions were tested, an additional control consisting of iodoacetamide-containing buffer was included. The presence of iodoacetamide had no effect on the amount of radioactivity bound. For serum samples, the antibody titer was expressed as the reciprocal of the last positive dilution. For IgM fractions, the Norwalk antibody titer was expressed as the reciprocal of the last positive dilution multiplied by the quotient obtained by dividing the total IgM concentration (milligrams per deciliter) in the whole serum by the total IgM concentration in the pooled IgM fraction prepared from that serum. Expressed in this way, the Norwalk antibody titers of whole serum and pooled fractions are directly comparable.

RESULTS

Specificity of the assay for IgM antibody. IgM was obtained from whole serum by sucrose gradient ultracentrifugation. Material shown by radial immunodiffusion to contain IgM without traces of IgG was tested for anti-Norwalk blocking activity in an RIA of established sensitivity and specificity. Reduction of four test IgM fractions with dithiothreitol (Table 1) resulted in the abolition of RIA blocking activity, whereas the activity of similarly treated IgG fractions was unaffected.

IgM antibody response to experimental Norwalk virus challenge. Figure 1 illustrates the humoral immune response to Norwalk virus challenge of a volunteer with preexisting whole serum Norwalk antibody. This volunteer developed gastroenteritis during the first 2 days after challenge and recovered by day 3. The first available blood specimen was collected on day 4 and showed an increase in serum antibody to Norwalk virus to 1:3,200 from a prechallenge titer of 1:800; no detectable virus-specific IgM was present on day 4. IgM was first detected on day 7 and reached a peak titer of 1:2,028 on day 15. At 8.2 weeks after challenge, a low titer of IgM antibody was still detectable in this volunteer. After 1.3 weeks, the total serum antibody titer was maintained at 1:≤51,200.

The humoral immune response of another volunteer who was without preexisting antibody to Norwalk virus and was subjected to three separate virus challenges is illustrated in Fig. 2. After the initial challenge, this individual became ill, and 4 weeks later moderate to low levels of both whole serum and IgM antibody to Norwalk virus were detected. The IgM antibody disappeared by 24.7 weeks, but serum antibody remained until 121 weeks, the time of the second challenge. This volunteer again became ill upon

<table>
<thead>
<tr>
<th>Serum fraction</th>
<th>Norwalk RIA antibody titer</th>
<th>Untrated</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 IgM</td>
<td>858</td>
<td>&lt;50</td>
<td></td>
</tr>
<tr>
<td>2 IgG</td>
<td>≥20,685</td>
<td>≥20,685</td>
<td></td>
</tr>
<tr>
<td>2 IgG</td>
<td>534</td>
<td>&lt;50</td>
<td></td>
</tr>
<tr>
<td>3 IgM</td>
<td>5,376</td>
<td>5,376</td>
<td></td>
</tr>
<tr>
<td>4 IgG</td>
<td>1,514</td>
<td>&lt;50</td>
<td></td>
</tr>
<tr>
<td>4 IgG</td>
<td>2,330</td>
<td>2,330</td>
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</tr>
<tr>
<td>4 IgG</td>
<td>2,028</td>
<td>&lt;50</td>
<td></td>
</tr>
<tr>
<td>4 IgG</td>
<td>4,168</td>
<td>4,168</td>
<td></td>
</tr>
</tbody>
</table>

a Reducing agent, 0.01 M dithiothreitol.

b Titer expressed as reciprocal.
FIG. 1. Total serum and IgM antibody responses to Norwalk virus of an infected volunteer. Ab, Antibody.

FIG. 2. Total serum and IgM antibody responses to Norwalk virus of a volunteer inoculated with the virus on three separate occasions. Ab, Antibody.
TABLE 2. Association of IgG seroconversion with IgM seroconversion after experimental Norwalk virus challengea

<table>
<thead>
<tr>
<th>Challenge result</th>
<th>IgM responseb</th>
<th>IgG response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Illness</td>
<td>Yes</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>2</td>
</tr>
<tr>
<td>No illness</td>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>3</td>
</tr>
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</table>

a Serum tested at a 1:50 dilution by RIA.
b Fourfold or greater rise in titer to Norwalk virus by RIA.

rechallenge, and 4 weeks later much higher titers of both serum and IgM Norwalk antibodies were seen than had been seen after the first challenge when no preexisting antibody was present. Upon short-term rechallenge 9.4 weeks later, the subject did not become ill and also did not mount an immune response consisting of either whole serum or IgM antibody.

Association of IgM with IgG seroresponses. When the IgM- and IgG-containing serum fractions were analyzed separately for Norwalk-specific antibody, seroconversions in the two antibody classes coincided in most ill individuals (Table 2). Of 17 virus challenges which produced illness, 15 were followed by a fourfold or greater antibody rise in both IgM and IgG. The two remaining challenges resulted in an IgG seroresponse only. An IgM seroresponse was much less likely to occur in the absence of illness (Table 2). Of seven virus challenges (excluding short-term rechallenge) which did not result in illness, only one seroresponse in both IgM and IgG was seen. Three of the remaining six challenges resulted in an IgG response only, and three did not result in a seroresponse. No IgM or IgG response was seen after three short-term rechallenges of previously ill and IgM and IgG seroresponding volunteers (data not shown).

Effect of prechallenge serum antibodies on IgM seroresponses. The presence of prechallenge serum antibody as evidence of prior exposure to Norwalk virus was not associated with a lack of IgM response upon rechallenge (Table 3). Of 14 challenges resulting in illness where prechallenge Norwalk antibody was present, 13 resulted in an IgM response. Two of three illness-producing challenges in the absence of preexisting antibody also resulted in an IgM response. Total Norwalk serum antibody was not present before any of the seven non-illness-producing challenges studied (Table 3). The three short-term rechallenges occurred in the presence of preexisting serum antibody and did not result in an IgM response (data not shown). For the most part, the prechallenge Norwalk antibody (excluding short-term rechallenge) was of the IgG class; however, two individuals possessed, in addition to IgG, relatively low titers of prechallenge IgM (1:200 and 1:300). They both developed illness after virus challenge.

Prevalence and time course of IgM response. Of 14 individuals who became ill after challenge, all but 2 developed an IgM response. The two volunteers not developing an IgM response both mounted a relatively low (1:800) serum antibody response. Only one of these two subjects had prechallenge total serum antibody (1:50).

The time course for the development of anti-Norwalk IgM was studied by analysis of sequential serum samples available from individuals who became ill and developed an IgM response. Of six specimens from such subjects collected between 0.6 and 0.9 week after challenge, only one contained anti-Norwalk IgM. All 28 serum samples available from these volunteers between 1.0 and 21.9 weeks after challenge were IgM positive. One sample collected at 24.7 weeks was IgM negative. The next group of six available specimens from these subjects was collected between 80 and 180 weeks after challenge, and all lacked anti-Norwalk IgM. The geometric mean titer of IgM antibody (Fig. 3) was significantly higher for samples collected between 1.0 and 2.9 weeks after challenge than for the remaining samples collected at later times after challenge. The geometric mean titer for 1.0 to 2.9 weeks was 614 (218, 1,884), and that for 3.0 to 21.9 weeks was 203 (127, 325), with P < 0.05 by the Student t test. The values in parentheses represent the 95% confidence intervals.

DISCUSSION

This study of the IgM seroresponse to experimental challenge with Norwalk virus revealed that an IgM response occurred in volunteers who became ill, whether or not prechallenge total serum antibody was present. The peak IgM

TABLE 3. Association of preexisting Norwalk serum antibody with IgM seroconversion after experimental challenge with Norwalk virusa

<table>
<thead>
<tr>
<th>Challenge result</th>
<th>IgM responseb</th>
<th>Preexisting antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Illness</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>1</td>
</tr>
<tr>
<td>No illness</td>
<td>Yes</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>6</td>
</tr>
</tbody>
</table>

a Serum tested at a 1:50 dilution by RIA.
b Fourfold or greater rise in titer to Norwalk virus by RIA.
The IgM response to another viral pathogen infecting the gastrointestinal tract, hepatitis A virus, shows some similarities to the pattern described here for Norwalk virus IgM. After infection with hepatitis A virus, there is a rapid simultaneous rise of virus-specific IgG and IgM antibodies. The major portion of the immunoglobulin response from the onset belongs to the IgG class, which persists for years after infection. Specific IgM is demonstrable at the time of jaundice and persists in diminishing amounts for up to 6 months. Hepatitis A-specific IgM is also found in association with subclinical and anicteric infections. Both sucrose gradient fractionation in conjunction with an RIA blocking test and anti-human IgM methods have been used successfully to diagnose recent hepatitis A infection in a single serum sample based on the measurement of virus-specific IgM (7). IgM-specific serological testing has been useful in the investigation of disease outbreaks and in the retrospective identification of nonicteric index cases (8, 15).

Certain inherent differences between hepatitis A and Norwalk virus infections exist which currently leave uncertain the usefulness of IgM testing as a diagnostic procedure for outbreaks of Norwalk disease. Our studies in volunteers indicate that Norwalk-specific IgM is not associated with subclinical illness; therefore, such index cases could not be identified by this method. Whereas hepatitis A is considered to be a one-time infection followed by long-term immunity, in the case of Norwalk illness recurrent infection in susceptible individuals is the apparent rule. Furthermore, it is clear from this study that the IgM response to Norwalk virus is not restricted to the primary infection, unlike what seems to be the case for rotavirus. Further information about the diagnostic utility of IgM testing for Norwalk virus will be gained from studies of naturally occurring outbreaks of Norwalk virus illness.

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LITERATURE CITED


