Rotavirus-Specific Intestinal Immune Response in Mice Assessed by Enzyme-Linked Immunospot Assay and Intestinal Fragment Culture

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Primate rotavirus strain RRV and bovine strain WC3 or reassortants made between these animal viruses and human rotaviruses have been administered to infants as candidate vaccines. We compared RRV and WC3 in a murine model of oral infection. We determined the relative capacities of these viruses to induce a virus-specific humoral immune response by intestinal lymphocytes as tested by enzyme-linked immunospot assay, intestinal fragment culture, and enzyme-linked immunosorbent assay of intestinal contents. We found that inoculation of mice with RRV induced higher frequencies of virus-specific immunoglobulin A (IgA)-secreting cells in the lamina propria, greater quantities of virus-specific IgA in intestinal fragment cultures, and greater quantities of virus-specific IgA in intestinal secretions than did inoculation with WC3 or inactivated RRV (iRRV). The induction of an IgA response in serum was predictive of an IgA response among intestinal lymphocytes after inoculation with RRV but not WC3. In addition, large quantities of IgG, IgA, and IgM not specific for rotavirus were produced in fragment cultures from mice inoculated with RRV but not in cultures from mice inoculated with WC3 or iRRV. Possible mechanisms of RRV-induced polyclonal stimulation of intestinal B cells are discussed.

Rotaviruses are recognized as the major cause of acute diarrheal disease in infants and young children (51); they are responsible for an estimated 830,000 deaths per year (20). Rotavirus infections are highly contagious and are difficult to control by general hygienic measures. Therefore, active immunization may be the most successful way to prevent disease. Because rotaviruses replicate only in mature villus epithelial cells which line the small intestine (45), protection against rotavirus disease is probably mediated by an immune response active at the intestinal mucosal surface. Development of a successful vaccine will depend on understanding the most efficient means of inducing virus-specific effector cells within gut-associated lymphoid tissue (GALT).

Efforts to develop a vaccine have focused on two animal-origin rotavirus strains which have attenuated virulence characteristics in humans: primate strain RRV (17) and bovine strain WC3 (8). In addition, reassortant viruses consisting of all gene segments from either RRV (7, 15, 17, 29, 39, 40, 50) or WC3 (5, 8, 9, 11, 53) and a single gene from human rotavirus strains have been developed and tested for efficacy. There are important differences between these two animal-origin rotavirus strains. First, RRV appears to be better adapted to growth in the human intestine than is WC3 (49). RRV is detected in the feces of infants and young children at higher titers and for longer periods than is WC3 (4, 5, 29, 40, 48). In addition, children inoculated with RRV develop fever more frequently than do children inoculated with either WC3 or placebo (4, 5, 7, 8, 28, 33, 48). Second, although studies directly comparing the immune responses to RRV and WC3 are lacking, WC3 may be less consistent than RRV in inducing a virus-specific immunoglobulin A (IgA) response in the serum and feces of infants (9, 28, 29, 40, 50).

As in humans, primate-origin rotaviruses are better adapted to growth in the murine intestine than are bovine-origin strains (3). Therefore, studies of the rotavirus-specific immune response in mice may be predictive of the immune response in humans. In this report we compare the intestinal virus-specific humoral immune response in mice after oral inoculation with rotavirus strains RRV and WC3 by using enzyme-linked immunosorbent (ELISPOT) assay, intestinal fragment culture, and enzyme-linked immunosorbent assay (ELISA) of intestinal contents.

MATERIALS AND METHODS

Mice. Conventionally bred, pregnant CD2 (F1) mice (Taconic Breeding Laboratories, Germantown, N.Y.) were housed in separate isolation units. Only litters from rotavirus-seronegative dams were used in these experiments.

Cells. Fetal green monkey kidney cells (MA-104 cells) were grown as previously described (36).

Viruses. Bovine rotavirus WC3 was obtained from H. Fred Clark, Philadelphia, Pa. Simian rotavirus RRV (MMU 18006) was obtained from Nathalie Schmidt, Viral and Rickettsial Disease Laboratory, Berkeley, Calif. Viral growth, purification, and quantitation by plaque assay were performed as previously described (36, 37).

RRV was inactivated by treatment with psoralen plus UV irradiation (19). 4′-Aminomethyl-4,5,8-trimethylpsoralen (HR1 Associates Inc., Concord, Calif.) dissolved in 50% ethanol was added at a final concentration of 10 μg/ml to a tissue culture-derived, clarified preparation of RRV. The preparation was placed on ice for 5 min, aliquoted into individual wells of a 24-well plate (Becton Dickinson and Co., Lincoln Park, N.J.),

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and exposed to a UV lamp (GBL-100; Geroge W. Gates & Co., New York, N.Y.) at a distance of 7.5 cm for 15 min.

Immunization of animals. CD2 (F1) suckling mice, 7 to 8 days old, were orally inoculated, by proximal esophageal intubation, with Stoker's medium (mock infection), inactivated RRV (iRRV) (215 ng per mouse), or tissue culture-derived WC3 or RRV (each at 10^7 PFU [approximately 215 ng] per mouse) in a volume of 100 μl.

ELISPOT assay. Lymphocytes from Peyer's patches (PP), mesenteric lymph nodes (MLN), small intestinal lamina propria (LP), and spleens were obtained 21 days after inoculation as previously described (38).

The ELISPOT assay was a modification of published methods (34, 43). Individual wells of 96-well millipore HA plates (Millipore Corp., Bedford, Mass.) were coated overnight at 4°C with 100 μl of phosphate-buffered saline (PBS) or 200 ng of purified, double-shelled RRV or WC3 suspended in 100 μl of PBS. The wells were washed four times with PBS (Wellschell 2; Denley Instruments Inc., Durham, N.C.) and treated for 1 h at room temperature (RT) with 100 μl of 1% bovine serum albumin (BSA) diluted in PBS. Wells were washed four times with PBS, and 100 μl of a suspension containing 1 × 10^6, 2 × 10^6, or 4 × 10^6 cells diluted in RPMI 1640 plus 10% fetal bovine serum was added to each well. The plates were incubated for 4 h at 37°C in a CO2 incubator and then washed five times with PBS and five times with PBS–0.05% Tween 20 (PBS-Tween) (Sigma Chemical Co., St. Louis, Mo.). Rotavirus-specific mouse IgGs were detected with isotype-specific, horse-radish peroxidase-labeled goat anti-mouse IgG (diluted 1:1,000 in 1% BSA–0.05% Tween), goat anti-mouse IgA (diluted 1:2,000 in BSA–0.05% Tween), or goat anti-mouse IgM (diluted 1:1,000 in BSA–0.05% Tween) (Southern Biotechnology Associates, Inc., Birmingham, Ala.). The plates were incubated overnight at 4°C in a humidified environment.

They were then washed four times with PBS, and 100 μl of a solution containing 10 μg of 3-amino-9-ethyl carbazole (Sigma) dissolved in 1 ml of N,N-dimethyl formamide (Sigma), 0.1 M sodium acetate (pH 5.0) (filtered through a 0.45-μm-pore size filter [Nalge Co., Rochester, N.Y.]), and 1% H2O2 (Sigma) was added to each well. After 15 min, the wells were washed with distilled water. The plates were dried for 24 h at RT, and the wells were examined by light microscopy (MZ8200 microscope; Swift, San Jose, Calif.) under ×8 to ×10 magnification. Average numbers of spots in duplicate wells coated without antigen were subtracted from average numbers in duplicate wells coated with antigen.

Intestinal fragment cultures. Intestinal fragment cultures were prepared 7, 14, and 21 days after inoculation, by a modification of the method described by Logan et al. (27). PP, MLN, and small intestinal fragments were collected under sterile conditions and washed three times in calcium- and magnesium-free Hank's balanced salt solution (Gibco Inc., Grand Island, N.Y.) supplemented with 10 mM N-2-hydroxy-ethylplperazine-N'-2-ethanesulfonic acid (HEPES) and 50 μg of gentamicin (JRH, Lenexa, Kan.) per ml. On average, 10 to 12 PP were collected per mouse. Each PP was incubated in one well of a 24-well plate (Becton Dickinson), suspended in 1 ml of a medium containing Kenne's HY medium (JRH) supplemented with 10 mM HEPES, 10% fetal bovine serum (Gibco), 4 mM L-glutamine (JRH), 100 μM of penicillin (JRH) per ml, 100 μg of streptomycin (JRH) per ml, 50 μg of gentamicin (JRH) per ml, and 0.25 μg of amphotericin B (Fungizone; JRH) per ml (GALT medium). MLN were incubated in Iscove's modified Dulbecco's medium (Gibco) supplemented with 50 μg of gentamicin per ml. After removal of the mesentery, connective tissue, and fat, MLN were cut in half and transferred to two wells of a 24-well plate containing 1 ml of GALT medium per well. After the PP were removed, the small intestine was opened longitudinally and cut into 2-in. (5-cm) segments. Small intestinal fragments were washed twice in HBSS, once in HBSS plus 0.05% EDTA (Sigma), and twice in HBSS. Eight small intestinal fragments (each approximately 0.25 cm long) were each placed in individual wells of a 24-well plate containing 1 ml of GALT medium per well. All tissue specimens were incubated for 7 days at 37°C in an atmosphere of 95% O2 to 5% CO2. Supernatant fluids (750 μl) were collected from each well, stored at −20°C, and tested by ELISA for detection of rotavirus-specific antibodies.

Each fragment containing LP cells had a surface area of 1 mm², whereas the total surface area of LP in the small intestine of suckling mice was calculated to be 1,400 mm². We adjusted the quantities of virus-specific and total antibodies obtained from eight small intestinal fragments to reflect antibody production by the entire small intestine.

Collection of intestinal secretions. Mice were fasted for 16 to 18 h prior to collection of intestinal fluids. A 500-μl portion of a solution containing 1.36 g of Golytel (Braintree Laboratories Inc., Braintree, Mass.), 2 ml of distilled water was inoculated orally by proximal esophageal intubation into each mouse four times at 15-min intervals. After 30 min, 150 μl of 1% pirocarnine hydrochloride (Sigma) was injected intraperitoneally and intestinal contents were collected in a petri dish containing 6 ml of PBS, 0.05 M EDTA (pH 7.4), and 0.1 mg of soybean trypsin inhibitor (type I-S; Sigma). Suspensions were vortexed for 30 s and centrifuged at 700 × g for 10 min at 4°C.

Then 60 μl of 100 mM phenylmethylsulfonfyl fluoride (PMSF; Sigma) in PBS was added to each sample. Samples were centrifuged at 27,000 × g for 20 min at 4°C; 4 ml was collected from each sample, and 40 μl of 2% sodium azide (Fisher Scientific Co., Pittsburgh, Pa.) and 40 μl of 100 mM PMSF were added. The samples were placed on ice for 15 min, and 200 μl of PBS and 1.4 ml of glycerol (Sigma) were added. The samples were mixed, divided into 1-ml aliquots, and stored at −70°C.

ELISA for detection of the titer of rotavirus-specific antibodies and quantities of IgA and IgG. Supernatant fluids from intestinal fragment cultures, supernatant fluids from intestinal contents, and serum from inoculated and uninoculated animals were tested for the presence of rotavirus-specific antibodies or quantities of total IgA, IgM, and IgG by ELISA. For detection of rotavirus-specific antibodies, individual wells of 96-well, flat-bottomed plates were coated with either 100 μl of PBS or 200 ng of purified WC3 or RRV diluted in 100 μl of PBS. The plates were stored overnight at 4°C. The wells were washed four times with PBS and blocked for 1 h with 200 μl of 0.25% BSA plus 0.025% Tween diluted in PBS at RT. The wells were then washed four times with PBS, 100 μl of sample was added to each well, and the mixture was incubated for 1 h at RT. The wells were washed four times with PBS, and 100 μl of horseradish peroxidase-conjugated goat anti-mouse IgM, IgA, and IgG (Southern Biotechnology Associates) diluted 1:2,000 in 1% BSA was added to each well and incubated for 1 h at RT. The wells were washed four times in PBS, and 100 μl of a 0.04% tetramethylbenzenedine peroxidase solution (Kirkegaard and Perry, Gaithersburg, Md.) was added; the mixture was incubated for 5 min. A 100-μl portion of 85% o-phosphoric acid (Sigma) was added to each well, and colorimetric changes were determined at an optical density (OD) of 450 nm on a microplate ELISA reader (Microplate reader 200; BioWhittaker, Walkersville, Md.). Samples were considered positive if OD values for virus-coated wells were both 0.1
unit greater and at least twofold greater than OD values for PBS-coated wells.

For detection of the quantities of total IgA and IgG in intestinal fragment cultures, wells were initially coated with 100 μl of PBS or a 1:1,000 dilution of either goat anti-mouse IgM, IgG, or IgA (Cappel, Organon Teknika Corp., West Chester, Pa.) diluted in PBS. As described above, 10 to 12 PFU were collected per mouse and plated in individual wells, while MLN were divided into two portions and placed in two wells. The individual PP and MLN OD values were added; their sums represented the total PP and MLN antibody output per animal. For small intestinal fragments, the sum of the OD values from eight LP samples was divided by 8 and multiplied by 1,400 (one fragment represents approximately 1/1,400th of the surface of the small intestine). Quantities of both virus-specific and total IgG, IgA, and IgM in supernatant fluids from intestinal fragment cultures and fecal contents were determined by comparison of values with those obtained from a standard curve constructed for each isotype with purified mouse IgM, IgG, and IgA (Sigma).

RESULTS

Frequencies of virus-specific plasma cells assessed by plasma cell ELISPOT assay. The specificity and sensitivity of our plasma cell ELISPOT assay were assessed by using hybridoma cells directed against either RRV inner capsid protein vp6 (IgG1; provided by Robert Shaw, Department of Veterans Affairs Medical Center, Northport, N.Y.) or influenza virus H1 hemagglutinin (IgG1; provided by Andrew Caton, The Wistar Institute of Anatomy and Biology, Philadelphia, Pa.). Similar to previous studies (34), 80 to 100% of putative antibody-secreting cells (ASC) were detected in wells coated with RRV and incubated with hybridoma cells secreting antibodies directed against RRV. No ASC were detected in wells coated with PBS and incubated with hybridoma cells directed against RRV or in wells coated with RRV but incubated with influenza virus-specific hybridoma cells. Cycloheximide treatment of mononuclear cells reduced the number of rotavirus-specific ASC by more than 90%, indicating that antibody secretion occurred de novo (data not shown).

We determined frequencies of rotavirus-specific plasma cells 3 weeks after oral inoculation of mice with 10⁷ PFU of either WC3 or RRV. The frequencies of virus-specific plasma cells bearing IgA in the LP, spleen, PP, and MLN were approximately 50- to 80-fold greater after inoculation with RRV than with WC3 (Table 1). There were no detectable rotavirus-specific ASC after mock infection of animals (data not shown). The total number of IgA-bearing cells in the LP of RRV- or mock-infected animals was 77,500 and 89,250 per 10⁶ mononuclear cells, respectively. Therefore, the increase in the number of rotavirus-specific ASC in LP was not accompanied by an increase in the total number of IgA-bearing cells. Rotavirus-specific ASC in the LP of mice inoculated with RRV accounted for approximately 14% of all IgA-bearing cells.

Quantities of total and virus-specific IgG produced in intestinal fragment culture. The quantities of total and virus-specific antibodies in supernatant fluids from intestinal fragment cultures of suckling mice were determined 7, 14, and 21 days after oral inoculation with iRRV (215 ng per mouse), 10⁷ PFU of WC3 (215 ng per mouse), or 10⁷ PFU of RRV (215 ng per mouse).

All of the groups tested showed an increase in total antibody production (IgM, IgA, and IgG) between 7 and 21 days after inoculation (Fig. 1D). In addition, greater quantities of total IgG, IgA, and IgM were produced by lymphocytes obtained from mice inoculated with RRV (from all tissues and at all time points) than in mice inoculated with iRRV or WC3 (Fig. 1D).

Virus-specific IgM was detected in the PP and MLN but not the LP of mice inoculated with RRV, WC3, and iRRV (Fig. 1A). Quantities of virus-specific IgA (and the percentage of virus-specific IgA compared with total IgA) obtained 21 days after inoculation were significantly larger in LP, MLN, and PP of RRV-infected mice than of WC3- or iRRV-infected mice (Fig. 1B). A total of 15% of IgG produced in fragment cultures of PP 21 days postinfection with RRV were rotavirus specific, compared with 1.74% of IgA produced in fragment cultures with WC3 or iRRV, respectively. Quantities of total and virus-specific IgG were smaller than for either IgA or IgM; no significant differences in virus-specific IgG secretion were observed among animals inoculated with RRV or WC3 (Fig. 1C). There were no differences in virus-specific or total antibodies secreted by PP taken from the proximal, medial, or distal segments of the small intestine (data not shown). Virus-specific IgM, but not virus-specific IgA and IgG, was detected in GALT of mice inoculated with iRRV (Fig. 1A to C), and neither virus-specific IgG, IgA, nor IgM was detected in GALT of mock-infected mice (data not shown).

Quantities of virus-specific antibodies in feces and serum. Rotavirus-specific antibodies were detected in the sera of animals 3 weeks after oral inoculation with RRV, WC3, or iRRV (Table 2). Circulating rotavirus-specific IgA was detected only in mice infected with RRV. High titers of rotavirus-specific IgG were detected in mice inoculated with RRV or WC3 but not in mice inoculated with iRRV. Rotavirus-specific IgM was detected in animals inoculated with RRV, WC3, or iRRV.

Rotavirus-specific and total IgA in intestinal contents were determined in animals 2 months after oral inoculation with RRV, WC3, or iRRV (Table 3). Rotavirus-specific IgA was detected in intestinal contents of animals inoculated with either RRV or WC3 but not iRRV. The quantity of rotavirus-specific IgA compared with total IgA was approximately fivefold greater after inoculation with RRV than with WC3.

<table>
<thead>
<tr>
<th>Immunizing strain</th>
<th>Source of lymphocytes</th>
<th>Frequency of virus-specific ASCs*&lt;br&gt;ng</th>
<th>IgA</th>
<th>IgG</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WC3</td>
<td>Spleen</td>
<td>3 ± 1</td>
<td>1 ± 0.5</td>
<td>50 ± 17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MLN</td>
<td>1 ± 0.5</td>
<td>4 ± 1</td>
<td>3 ± 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PP</td>
<td>18 ± 11</td>
<td>2 ± 0.5</td>
<td>31 ± 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LP</td>
<td>158 ± 64</td>
<td>ND³</td>
<td>20 ± 16</td>
<td></td>
</tr>
<tr>
<td>RRV</td>
<td>Spleen</td>
<td>97 ± 12</td>
<td>5 ± 2</td>
<td>58 ± 32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MLN</td>
<td>85 ± 17</td>
<td>3 ± 1</td>
<td>ND³</td>
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<tr>
<td></td>
<td>PP</td>
<td>1,014 ± 82</td>
<td>71 ± 19</td>
<td>6 ± 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LP</td>
<td>10,668 ± 938</td>
<td>11 ± 4</td>
<td>ND³</td>
<td></td>
</tr>
</tbody>
</table>

* Groups of three 7- to 10-day-old CD2(F1) suckling mice were orally inoculated with 10⁷ PFU of either tissue culture-derived bovine strain WC3 or primate strain RRV. At 3 weeks later, animals were sacrificed and lymphocyte populations were obtained to determine the frequencies of rotavirus-specific ASC.

* Frequencies of virus-specific ASCs are shown per 10⁶ mononuclear cells. Standard errors were calculated on samples performed in duplicate wells.

* ND, not detected at frequencies of 1 rotavirus-specific ASC per 4 × 10⁶ mononuclear cells.
TABLE 2. Titers of rotavirus-specific antibodies in serum by ELISA after oral inoculation of mice with RRV, WC3, or iRRV<sup>a</sup>

<table>
<thead>
<tr>
<th>Immunizing strain</th>
<th>Time (days) after immunization</th>
<th>Titer of rotavirus-specific antibodies&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgM</td>
</tr>
<tr>
<td>WC3</td>
<td>7</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>400</td>
</tr>
<tr>
<td>RRV</td>
<td>7</td>
<td>3,200</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1,600</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>3,200</td>
</tr>
<tr>
<td>iRRV</td>
<td>7</td>
<td>3,200</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1,600</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>1,600</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sera were obtained from groups of two animals 7, 14, and 21 days after oral inoculation with either 10<sup>7</sup> PFU of RRV (215 ng of virus protein), 10<sup>7</sup> PFU of WC3 (215 ng of virus protein), or 215 ng of iRRV.

<sup>b</sup> Data are shown as the reciprocal of the highest serum dilution positive by ELISA and represent the average result for sera obtained from two animals.

DISCUSSION

Each of the three assays chosen for these studies addresses a selected aspect of the intestinal humoral response. The ELISPOT assay enables one to determine the frequencies of virus-specific plasma cells (of specific isotype) within a given population of cells. However, this assay does not necessarily predict the quantity or isotype of virus-specific antibodies located at the intestinal mucosal surface, nor does it allow for detection of virus-specific neutralizing antibodies. Intestinal fragment culture preserves the native microenvironment within GALT and allows for determination of the quantities, isotypes, and neutralizing capacities of antibodies produced by PP and LP. However, production of antibodies in vitro is not necessarily predictive of events occurring at the intestinal mucosal surface. ELISA of intestinal contents allows for a direct determination of the presence of virus-specific antibodies at the intestinal mucosal surface. Unfortunately, measurement of the quantity of virus-specific secretory IgA (sIgA) in intestinal fluids may be confounded by degradation of sIgA by intestinal proteases, entrapment of sIgA in mucus, and variable dilution of sIgA by the osmotic catharsis required for collection. For these reasons, the three assays chosen complement each other in their capacity to detect the rotavirus-specific humoral immune response in GALT.

Inoculation of mice with RRV induced significantly greater frequencies of rotavirus-specific IgA-secreting cells in LP, MLN, and PP as determined by ELISPOT assay than did inoculation with either WC3 or iRRV. Similarly, RRV induced secretion of greater quantities of rotavirus-specific IgA by PP and LP in fragment cultures and greater quantities of rotavirus-specific IgM, IgA, and IgG responses were combined (D). Ab, antibody.

FIG. 1. Ig responses in intestinal fragment cultures. CD<sub>2</sub> (F<sub>i</sub>) suckling mice (7 to 8 days old) were orally inoculated with either 10<sup>7</sup> PFU of RRV (215 ng per mouse), 10<sup>7</sup> PFU of WC3 (215 ng per mouse), or 215 ng of iRRV per mouse. Groups of two mice were sacrificed 7, 14, and 21 days after inoculation, and intestinal fragment cultures were established for PP, MLN, and LP of the small intestine. Supernatant fluids from these cultures were tested by ELISA for the presence of rotavirus-specific and total IgM (A) IgA (B), and IgG (C). Total and virus-specific IgM, IgA, and IgG responses were combined (D). Ab, antibody.
virus-specific IgA in intestinal contents than did WC3 or iRRV. Oral inoculation of mice with WC3 induced low frequencies of virus-specific IgA-secreting cells by ELISPOT assay; this finding correlated with the small quantities of IgA secreted by GALT in fragment cultures and the low levels of virus-specific IgA in intestinal contents. The induction of a vigorous, virus-specific IgA response in GALT by RRV probably occurs because RRV is better adapted to growth at the murine intestinal mucosal surface than is WC3 (3). The importance of RRV replication in induction of a virus-specific IgA response in GALT is supported by the finding that noninfectious RRV does not induce virus-specific IgA. Our findings are similar to those of VanCott et al. (46), who found that the capacity of porcine coronaviruses to induce virus-specific IgA-producing ASC in GALT after oral inoculation was related to the virulence of the immunizing strain. Virus replication in intestinal epithelial cells (which express class II antigens [6]) may cause antigen to be presented in a manner more likely to induce secretion of cytokines associated with production of IgA (i.e., interleukin-5, IL-5 and IL-6 [1, 2]) than does virus which does not replicate. Alternatively, induction of a virus-specific IgA response in GALT may be related simply to the amount of antigen presented to the intestinal mucosal surface. The quantity of RRV (but not WC3) presented to the intestinal mucosal surface is amplified by replication in intestinal epithelial cells (3). Therefore, it may be possible to inoculate mice with a quantity of WC3 or iRRV sufficient to induce a vigorous, virus-specific IgA response.

We found that approximately 14% of all IgA-bearing cells in the LP of suckling mice inoculated with RRV were rotavirus specific. These findings are similar to those previously reported for suckling mice orally inoculated with RRV or murine rotavirus (16, 34). It is known that at the age of 10 days very few IgA plasma cells are present in the LP of suckling mice. However, by 2 weeks of age, there is a dramatic increase in the number of IgA plasma cells (47). The large percentage of rotavirus-specific plasma cells may be due to the virus being the first foreign antigen that the mucosal immune system sees, and hence the response is vigorous.

The presence of rotavirus-specific IgA in serum paralleled the presence of a rotavirus-specific IgA response in GALT detected by ELISPOT assay, intestinal fragment culture, and ELISA of intestinal contents. However, a WC3-specific IgA response detected by intestinal fragment culture and by ELISA of intestinal contents was not detected in serum. Serum antibodies may not parallel the immune response occurring at the intestinal surface. Our findings with RRV-inoculated mice are similar to those of a number of studies with mice and humans in which a correlation was demonstrated between antigen-specific ASC in GALT or antigen-specific antibodies in intestinal contents and the presence of antigen-specific antibodies in the circulation (12, 14, 18, 22, 41, 52). These findings are consistent with the observation that antigen-specific ASC are induced in PP and travel via the circulation to the lamina propia (13).

The titers of virus-specific IgG induced in the circulation and the quantities of virus-specific IgG produced by GALT in fragment culture were similar after inoculation of mice with RRV or WC3. However, the frequencies of virus-specific IgG-secreting plasma cells in GALT by ELISPOT assay after RRV or WC3 inoculation were low. These findings suggest that intestinal fragment culture is a more sensitive assay for detection of virus-specific, IgG-secreting cells in GALT than the ELISPOT assay is (and that the virus-specific IgG response detected in serum is predictive of a virus-specific IgG response in GALT). In support of this hypothesis, virus-specific IgG was not detected in either serum or fragment cultures of mice orally inoculated with iRRV; similarly, virus-specific IgM was detected in both serum and intestinal fragment cultures. In addition, Ward et al. (52) found a correlation between the presence of rotavirus-specific IgG in serum and jejunal fluid in adults orally inoculated with a human rotavirus strain. Alternatively, virus-specific IgG may be made at sites distant from the intestine after oral inoculation with RRV or WC3. Antigen-presenting cells in PP may transport and present antigen to T cells at sites other than the intestine (e.g., the spleen). For example, Liu and MacPherson (25, 26) found that dendritic cells in PP and LP can acquire antigen after oral administration and present antigen to primed splenic lymphocytes. In addition, infectious virus or viral antigens may enter the circulation, travel to the spleen, and stimulate a virus-specific IgG response. Rotavirus antigen has been detected in the PP and MLN of mice (16), and infectious virus has been detected in the MLN of cows (32), after homologous host infection.

Production of large quantities of IgG, IgA, and IgM not specific for rotavirus was observed in intestinal fragment cultures from mice orally inoculated with RRV but not in fragment cultures from mice inoculated with WC3 or iRRV. Increased secretion of antibodies was not associated with an increase in the frequencies of IgA-bearing cells in GALT in mice inoculated with RRV as compared with unoinoculated animals. Similarly, bystander stimulation of GALT antibody-secreting cells occurs in mice orally inoculated with reovirus type 1 (24a). There are several possible hypotheses to explain these observations. First, infection with RRV may induce synthesis of cytokines associated with the Th2 phenotype (e.g., interleukin-4 [IL-4], IL-5, and IL-6) in LP. It has been shown that IL-5 and IL-6 are important in the induction of IgA synthesis from B cells already committed to IgA synthesis (1, 2) and that the LP of adult mice is rich in Th2 cells (31). We are presently determining the capacity of RRV or WC3 to induce Th1 or Th2 cells in GALT after oral inoculation of mice. Second, RRV replication in intestinal epithelial cells, as with transmissible gastroenteritis virus, may cause disruption of the mucosal surface (24); penetration of commensal bacterial, fungal, or environmental antigens; and stimulation of antigen-specific antibody responses. Third, replication of RRV within intestinal epithelial cells may break the oral tolerance to dietary or microbial antigens, as in the case with inflammatory bowel disease (21, 54) which results in the production of autoantibodies and antibodies against environmental antigens. Finally, RRV, like human immunodeficiency virus (44) and Epstein-Barr virus (23, 42), causes a bystander B-cell polyclonal stimulation.

The immunologic response which clearly correlates with protection against rotavirus disease has not been determined. Several studies found that the presence of rotavirus-specific

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amt of total sIgA (ng/ml)</th>
<th>Amt of virus-specific sIgA (ng/ml)</th>
<th>Virus-specific IgA/total IgA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iRRV</td>
<td>19,000</td>
<td>&lt;4</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>WC3</td>
<td>23,000</td>
<td>41</td>
<td>0.18</td>
</tr>
<tr>
<td>RRV</td>
<td>15,000</td>
<td>148</td>
<td>1.02</td>
</tr>
</tbody>
</table>

* Intestinal contents were obtained 2 months after oral inoculation of suckling mice with either 10⁶ PFU of RRV (215 ng of virus protein), 10⁷ PFU of WC3 (215 ng of virus protein), or 215 ng of iRRV. Intestinal contents were pooled from two mice in each group.
IgA in serum or feces correlated with protection against challenge (12, 14, 18, 30, 52). We found that induction of rotavirus-specific IgA responses among GALT lymphocytes in mice by RRV was better than that induced by WC3. Similarly, WC3 may be less consistent than RRV in inducing virus-specific IgA responses in the serum and feces of infants (9, 28, 35). However, the capacity of RRV to protect against rotavirus disease in vaccine trials is not clearly different from that of WC3 (9, 10, 40). Either rotavirus-specific IgA alone is not predictive of protection against disease or RRV and WC3 do not differ in their capacity to induce a virus-specific IgA response in infants. A study comparing the relative capacities of RRV and WC3 to induce a virus-specific IgA response in infants remains to be performed.

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