Murine Intestinal Antibody Response to Heterologous Rotavirus Infection

AYUB A. MERCHANT, WILLIAM S. GROENE, EDWARD H. CHENG, AND ROBERT D. SHAW*  

Department of Medicine, Northport Veterans Medical Center, Northport, New York 11768, and Department of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11790

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Rotavirus is the most important worldwide cause of severe gastroenteritis. Extensive efforts have been devoted to the design of a vaccine that will prevent disease, but development of a more effective vaccine strategy may require progress in the understanding of the mucosal immune response to replicating viral antigens. In this article, we report the characterization of the intestinal antibody response of a murine model to heterologous infection with the rhesus rotavirus vaccine strain. We have adapted the enzyme-linked immunospot assay to measure this response without the difficulties associated with measurement of antibodies in intestinal contents or the artifacts associated with culturing of lymphocytes. The predominant response in terms of antibody-secreting cells (ASC) is seen in the small intestine lamina propria, which can be measured within 4 days of infection, peaks 3 weeks after infection, and remains near that level for longer than 8 weeks. The magnitude of the immunoglobulin A (IgA) cell response is approximately 10 times greater than the intestinal IgG cell response, and IgM cells are rare. Virus-specific ASC constitute approximately 50% of all ASC in the gut at the peak of the virus-specific response. This response is considerably greater than responses to nonreplicating mucosal antigens measured by similar techniques. Enteral infection engenders minimal virus-specific ASC response in the spleen. Rhesus rotavirus-specific enzyme-linked immunosorbent assay and neutralization assays of serum and intestinal contents did not correlate with virus-specific ASC response.

Rotavirus is an important cause of severe gastroenteritis in humans, as well as in other mammalian and avian species (15, 26). In children under the age of two years, it is the single most important cause of severe diarrhea and contributes significantly to infant mortality in less developed countries (29, 56). Although death due to rotavirus gastroenteritis occurs rarely in developed countries, it is still an extremely common cause of morbidity and physician visits (20, 21). Rotavirus can also cause disease in adults, especially the elderly and the immunocompromised (22). The tremendous worldwide impact of this virus has led to efforts to control this disease by the development of an effective vaccine.

The development of efficient vaccine strategies may be dependent on an understanding of the host immune response to natural infections and vaccination. Immunity to rotavirus infection has been studied with humans and several animal models, but the precise nature of the host response to infection or vaccination is still not clear. Several studies have suggested the importance of local antibodies at the mucosal surface of the small intestine as a major determinant of resistance to rotavirus illness (3, 5, 31, 39, 40, 49, 52, 55). Serum, feces, duodenal aspirate, saliva, and breast milk antibody titers have all been measured by using various immunoassays to define the antibody response. Unfortunately, most assays may not provide reliable measures of the intestinal response because of a variety of artifacts, including antibody degradation, mucus entrapment, dilution in secretions, or simply because of the inherent compartmentalization of the systemic and local antibody responses. Furthermore, the passive acquisition of antibodies from the mother in utero and later from breast milk can interfere with the quantitation of immunoglobulin production as measured in titers of serum or intestinal contents.

Studies on specific antibody responses at the mucosal level have been limited by technical difficulties of immuno-histochemical staining and plaque assays as well as inherent difficulties of quantitation in these assays. A modification of the enzyme-linked immunospot (ELISPot) technique has allowed us to accurately enumerate functionally active antibody-secreting cells (ASC) within intestinal tissues and define the local antibody production in terms of specific location, kinetics, antigen specificity, and isotype. Use of this assay directly on freshly isolated lymphocytes from the gut-associated lymphoid tissues without significant in vitro manipulations allows for the most accurate estimation of their in vivo activity.

Though various animal models have been developed to study the pathogenesis of and immune response to rotavirus infection, none represents a perfect system. The short gestation period, modest price, ease of isolation of animals, commercial availability of immunological reagents, and the well-characterized immune system make the suckling mouse a convenient small-animal model which has been widely used (16, 23, 35–38, 46). Moreover, the infection in suckling mice closely parallels that seen in human infants in pathogenesis, pathological lesions, and symptomatic but self-limiting diarrheal illness. For these reasons, we chose this model for the study of intestinal immune response.

The vaccine strategy most widely tested to date utilizes animal viruses that are naturally attenuated in humans (27). Rhesus rotavirus (RRV), a simian strain, has been effective at stimulating only type-specific immunity in various field trials (8, 17–19, 44, 45, 67). This rotavirus strain has previously been shown to replicate and cause disease in mice when administered at sufficient doses (43). Because of the extensive prior studies on murine and human infection and the substantial virulence of this rotavirus strain in a heterologous mouse model, we chose this well-characterized strain as the infecting agent.

* Corresponding author.
The present study defines the kinetics, isotype specificity, and the anatomy of the murine intestinal antibody responses to a single oral inoculum of the heterologous RRV strain. We also studied the antibody titers in the serum and intestinal contents in an attempt to seek correlation of ASC numbers with these previously described parameters.

MATERIALS AND METHODS

Animals and immunization. Pregnant CD2F1 hybrid mice (pathogen free) were obtained from Taconic Farms (Germantown, N.Y.) and housed in microisolator cages containing sterile bedding, food, and water throughout the experiment. Sera obtained from mice upon arrival were tested by focus neutralization assay to ensure no prior exposure to rotavirus. Litters were infected with RRV (ATCC VR 954) at the age of 9 to 12 days. The virus was grown in MA104 cells in serum-free M199 culture medium as previously described, released by freezing and thawing, and stored at \(-90^\circ C\) (25). The dose of virus administered by gastric intubation was 10^7 PFU administered in 100 \(\mu\)l of tissue culture supernatant. The virus was not trypsin activated in vitro prior to administration, nor were restrictions made to virus dose after food intake before and after immunization. This dose of virus resulted in diarrhea in more than 95% of pups, usually between 2 to 4 days after infection. Two littersmates were sacrificed at defined time intervals, and the spleen, mesenteric lymph nodes (MLN), small intestine, intestinal contents, and serum from each were obtained. Experiments included pups of both sexes whenever possible to minimize the significance of sex-specific influences on the immune response, if any. Naïve mice housed in adjacent cages showed no evidence of diarrhea or a specific immune response in parallel experiments, indicating the absence of cage-to-cage transmission of RRV. Sentinel mice were tested by serum immunofluorescence assay for the murine rotaviruses (which cause epizootic diarrhea of infant mice) by Ahmed Biosafe Inc. (Rockville, Md.) and were reported to test negative.

Intestinal fluid collection. The contents of the small intestine were collected by a method previously described, with minor modification (14). Briefly, after the small intestines were removed, the outside of each was rinsed twice with cold Hanks balanced salt solution, and about 5 ml of cold Hanks balanced salt solution was then rinsed through the inside of each intestine and mixed 1:1 with protease inhibitor solution (50 mM EDTA, soybean trypsin inhibitor [0.1 mg/ml], pH 7.4). After being vortexed for 1 min the tube was centrifuged at 850 \(\times\) g at 4°C for 10 min, and 100 \(\mu\)l of phenylmethylsulfonyl fluoride (100 mM in 95% ethanol) was added to the supernatant in a clean tube. The tube was then centrifuged at 1,300 \(\times\) g at 4°C for 25 min. One hundred microliters of phenylmethylsulfonyl fluoride, 0.01% sodiumazide, and 300 \(\mu\)l of fetal bovine serum (FBS) were added to the supernatant in a clean tube. The contents were aliquoted and frozen at \(-90^\circ C\).

Lymphocyte isolation procedure. Small intestinal contents were removed as described above, and the intestine was collated over a narrow spatula. Macroscopically visible Peyers patches (PP) were dissected. Lamina propria (LP) lymphocyte isolation was accomplished by a modification of the procedure first described by Davies and Parrott and modified by Van der Heijden et al., by using EDTA and collagenase, which allows recovery of functionally active intestinal lymphoid cells (12, 63). The cell suspension was subjected to a discontinuous Percoll gradient centrifugation to isolate viable mononuclear cells, which were enumerated by trypan blue exclusion. Spleen, PP, and MLN tissues were minced and forced through steel mesh; clumps were allowed to settle, and the supernatant was decanted. Splenic erythrocytes were lysed with 0.83% ammonium chloride. Mononuclear cells from these tissues could be reliably counted without Percoll gradient centrifugation.

ELISPOT. We utilized a modified version of the ELISPOT technique to measure the number of total and RRV-specific ASC of each isotype (immunglobulin G [IgG], IgA, and IgM) present in spleen, LP, PP, and MLN tissues (11). Immobilon PVDF (Millipore Corp., Bedford, Mass.), a white hydrophobic polyvinylidene difluoride membrane, was used as the protein-binding matrix. The membrane was mounted in a 16- or 28-lane minibliterator (Immunetics, Cambridge, Mass.). Lanes on the template-mounted membrane were coated with the desired capture antigens, which included RRV diluted in 0.01 M Tris, 0.01 M NaCl, and 2 mM CaCl_2, pH 7.5 (TNC buffer), or rabbit anti-mouse IgG, goat anti-mouse IgM, or goat anti-mouse IgA diluted in phosphate-buffered saline (PBS). The optimum concentrations for the capture antigen substances were previously determined in titration experiments with appropriate hybridoma cell supernatant. The sensitivity of the assay was determined by the use of cesium chloride gradient-purified rotavirus as the capture antigen and purified on cesium chloride density gradients as previously described (51). This template was incubated at 37°C for 2 h on a rocking platform. Lanes were washed with PBS to remove unbound capture antigen, and the membrane was blocked with PBS containing 5% FBS for 1 h at 37°C.

Lymphocyte cell suspensions in the appropriate dilutions in RPMI 1640 containing 2% FBS were made so that a countable number of spots resulted in each lane after a fixed volume of the cell suspension was incubated on the capture antigen for 2 h at 37°C (the optimal incubation time and cell concentrations were determined in preliminary experiments). Once the incubation was complete, the cells were washed from the membrane with PBS. Detection of bound mouse immunoglobulin was accomplished with isotype-specific biotinylated anti-mouse immunoglobulin (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) and avidin-horseradish peroxidase conjugate (Vector Labs, Burlingame, Calif.) by employing the precipitating substrate 3-aminono-9-ethylcarbazole (Sigma Chemical Co., St. Louis, Mo.), which resulted in red-brown spots. Figure 1 is a representative photograph of the ELISPOT assay. The spots were enumerated under \(\times 2\) to \(\times 6\) magnification with an Olympus SZ-FT zoom stereo microscope with a halogen fiber optic light source (by the same individual). Within experiments, multiple lanes of a particular type were run, and the mean was used to determine the total number of spots per 10^6 viable mononuclear cells. Very high numbers of spots per lane (>300) were difficult to count because of the coalescence of spots, and the results were taken from wells incubated with fewer cells.

ELISA. IgG and IgA isotype-specific antibodies to rotavirus were measured in the serum and intestinal contents by using an indirect double-sandwich enzyme-linked immunosorbent assay (ELISA). IgM ELISA was not performed. A guinea pig anti-RRV hyperimmune serum was developed, and IgG was affinity purified from this serum by the Membrane Affinity Separation System (Nygene Corp., Yonkers, N.Y.). This IgG was used as capture antibody on Immulon II microtiter plates (Dynatech Laboratories, Alexandria, Va.). The binding of cesium chloride gradient-purified rotavirus. Optimum dilution for the capture antibody and rotavirus was determined by checkerboard titration in preliminary studies.
to ensure adequate rotavirus binding and minimal nonspecific interactions. The plates were blocked with 2% FBS, and twofold dilutions of serum or intestinal content samples in PBS with 1% FBS were added and incubated for 2 h. Subsequently, the bound anti-rotavirus antibodies were detected by using biotinylated isotype-specific goat anti-mouse immunoglobulin and avidin-horseradish peroxidase conjugate (as above); o-phenylenediamine dihydrochloride was used as the soluble substrate (Sigma Chemical Co.) in a 0.4-nmol solution in citrate buffer, pH 5.3, and activated with 0.2 μl of 30% hydrogen peroxide per ml. The reaction was stopped after 10 min by the addition of 3 N sulfuric acid, and the A_{405} of the reactants was read on an EL309 automated microplate reader (Biotek, Winooski, Vt.). Parallel wells that were not coated with RRV were used as controls to quantitate nonspecific binding; control values were subtracted from those for the RRV-coated wells. All samples were run in triplicate, and titers are reported as the reciprocal of the highest dilution giving a mean absorbance reading of >0.10 optical density units.

**Immunohistochemical focus neutralization assay.** Serum and intestinal contents were assayed for RRV neutralization by an immunohistochemical focus neutralization assay as previously described (50). The neutralization titer was that dilution of the sample that resulted in >50% reduction of RRV antigen-positive MA104 cells.

**Statistical analysis.** Cells were harvested from spleen, PP, MLN, and small intestine LP tissues from animals 4 to 63 days after infection with RRV. These cells were independently assayed for secretion of total IgG, IgM, and IgA antibodies as well as specific antibodies for gradient-purified double-shelled rotavirus particles. The data from the 33 experiments were analyzed by grouping these experiments over five time intervals of 10, 20, 30, 45, and 60 days postinfection. Comparisons among means of groups were done by analysis of variance and multiple comparisons by the Scheffe F test by using StatView II (Abacus Concepts, Berkeley, Calif.) on a Macintosh computer. An additional nine experiments were done with naive mice 14 to 45 days after mock infection with virus-free tissue culture supernatants.

### RESULTS

**Specificity of ELISPOT.** The groups of naive or of mock-infected animals showed <33 ASC per 10^6 specific ASC of any one isotype in all tissues, except IgG and IgM in spleen, in which up to 133 ASC per 10^6 specific ASC were seen in some experiments. This phenomenon, previously described, is believed to be secondary to the secretion of cross-reactive antibodies against a variety of antigens, which is most marked in the spleens of 1- to 5-week-old mice during the acquisition and maturation of the B-cell repertoire (28). Hybridoma cells secreting rotavirus-specific antibodies against VP6, VP7, and VP4 (255/60, 4F8, and 7A12, respectively) were included as positive and negative controls in each experiment and consistently showed equivalent numbers of spots in the immunoglobulin and rotavirus capture lanes. Lanes coated with irrelevant antigens such as cholera toxin or no capture also showed <33 ASC per 10^6 specific ASC.

Cycloheximide treatment of the hybridoma cells prior to and during the incubation decreased the number of spots, indicating that the assay accurately defined active antibody secretion and did not detect membrane-bound fragments (10). At a concentration of 100 μg/ml, monoclonal antibody 4F8 hybridomas showed a 76% reduction in spot formation, and monoclonal antibody 7A12 hybridomas showed an 83% reduction.

**Sensitivity of ELISPOT.** The percentage of hybridoma cells mentioned above secreting IgG was 64 to 72%, varying with the condition of the cell culture at the time of the assay. The efficiency of RRV capture varied from 74 to 100% of the IgG-secreting cells. An IgM-secreting hybridoma, M14, was also tested and gave comparable numbers and quality of spots, suggesting a comparable sensitivity of this assay to detect these immunoglobulin isotypes. In addition, we report equivalent numbers of IgG and IgM secretors in the spleen. This is consistent with previously described data and indicates an equal sensitivity for the two isotypes (64). As IgA hybridomas against RRV are not generally available, we were not able to test the sensitivity of IgA ASC detection with the hybridoma cells. However, the large number of IgA-secreting cells in the LP attest to the sensitivity for detection of that isotype and correlate with the numbers found by other investigators (see Discussion).

**Total or background ASC.** The numbers of ASC of each isotype in the four tissues studied are shown in Table 1. This table includes all time points beyond day 10 postinfection, as there was no significant difference in their numbers over the duration of these experiments. The LP showed the greatest number of active ASC, predominantly of the IgA isotype. The total number of IgA-secreting cells of over 100,000 per 10^6 mononuclear cells was also reported by Van der Heijden et al. in 20-week-old mice (64). The number of ASC 10 days postinfection was significantly smaller than at all later times.
(see Fig. 3), probably reflecting the immaturity of the immune system at an age of 3 weeks or less as previously described (1).

Figure 2 shows a comparison of the ASC (IgG, IgM, and IgA) in the small intestine LP tissue from mice that were enterally infected with rotavirus with similar cells from mock-infected animals. The marked rise of IgA ASC between 15 and 25 days of age (approximately 5 to 15 days postinfection) is approximately 7 days earlier than in the uninfected control animals. The data for uninfected is consistent with previous studies on murine intestinal IgA ontogeny (see Discussion) (1, 62). The relatively early development of a plateau may have been enhanced by the RRV infection, although that conclusion cannot be drawn from these data alone.

**RRV-specific ASC.** Figure 3a through d shows the mean (± standard error of the mean [SEM]) of total and rotavirus-specific ASC of each isotype, expressed as the number of ASC found per one million viable mononuclear cells derived from each of the four tissues studied at the specified time groups. The data from all time groups except day 10 postinfection are pooled in Table 2, which shows the mean number of ASC (± SEM) of each isotype in the four tissues studied.

In our experiments, the RRV-specific IgA responses in LP were evident by 4 days postinfection, rose to peak around 30 days postinfection, and persisted at high levels for up to 63 days postinfection. As seen in Fig. 3a, at the height of the RRV-specific response, it accounted for more than 50% of all IgA-secreting cells. This confirms that a single dose of this heterologous virus is highly immunogenic in the mucosal immune system of mice.

A rise in RRV-specific IgA activity was also seen in the PP, which correlated with the response in LP but was much smaller in magnitude (Fig. 3b). It is possible that this represented contamination of PP with LP cells, which was inevitable in spite of careful dissection. As expected, the spleen showed a predominance of IgM- and IgG-secreting cells, with little IgA activity. To be noted is the extremely small number of rotavirus-specific ASC of any isotype found in the spleen, a statistically significant difference from the small intestinal response. In the four experiments done at 4, 7, 8, and 11 days postinfection, significantly increased IgM and IgG responses was seen in the MLN (Fig. 3c). These early IgG and IgM responses were not seen in any other tissue and disappeared by 14 days postinfection.

**ELISA.** Serum and intestinal contents obtained from naive controls and the mice sacrificed on day 4 postinfection showed IgA titers of <1,000 and <50 and IgG titers of <500 and <25, respectively. A fourfold or higher rise up to 64,000 and 3,200 for IgA and up to 32,000 and 1,600 for IgG, respectively, was noted in animals sacrificed after day 14, and the mean titers for the different groups are shown in Fig. 4. The kinetics of the response are similar to those of the ASC by the ELISPOT technique. However, the peak serum IgG titer occurred at a later time, possibly reflecting the continued accumulation of IgG in the serum due to the long half-life of this immunoglobulin class.

When individual animals were compared, a fairly good correlation between serum and intestinal IgA titers was observed (r = 0.82). The correlation between the small intestinal and serum IgA titers may be related to the relatively efficient clearance of serum IgA by the hepatocytes into bile via a receptor-mediated pathway present in mice and some other rodents but not in humans (4, 57). However, neither correlated with the number of ASC in the LP (r = 0.40 for RRV-specific serum IgA versus LP IgA ASC and 0.53 for intestinal contents), indicating other possible variables that influence antibody titer in these fluids.

**Neutralization.** Serum and intestinal content samples were collected at the time of lymphocyte harvest and assayed for RRV neutralization titers by immunohistochemical focus reduction assay. The results for samples from 12 experiments from 10 to 60 days postinfection are shown in Fig. 5. While there is a suggestion of peaking titers 20 to 30 days
postinfection, no statistical trend can be identified. Neutralizing antibody titers in nonimmunized animals and mothers prior to their litters being immunized were always <200 in serum and <20 in the intestinal contents. A representative group of dams tested at the same time as the immunized mice were sacrificed and showed significant neutralizing titers approaching those seen in the immunized litter (data not shown). Since the mice immunized were of suckling age, the mothers were housed in the same cage and were therefore exposed to the virus shed in the feces of immunized litter. The possibility of passive transfer of neutralizing antibody from mothers, in the serum by immunoglobulin uptake before intestinal closure and in the intestinal lumen of suckling mice, makes the interpretation of these data difficult and emphasizes the advantage of ELISPOT assay.

**DISCUSSION**

Rotaviruses are a common cause of severe gastroenteritis and produce disease by replicating in the terminally differentiated epithelium of the small intestine. Several studies have indicated the importance of local mucosal immunity in the protection against this disease. Passive transfer of gamma globulins, neutralizing monoclonal antibodies, or antibodies in milk orcolostrum have been shown to be protective when administered together with infectious doses of viruses (3, 5, 31, 39, 40, 49, 52, 55). Antibodies in serum seem not to protect against infection in most studies (34, 54, 70). However, some studies have suggested that high titers of passive circulating antibodies may moderate the severity of rotavirus infections via possible resecretion into the intestine (48).
Rotavirus-specific plasma cells in the small intestine starting at 10 days postinfection have previously been demonstrated by Dharakul et al. by using immunohistochemical methods in a murine model of homologous rotavirus infection (13). Ninety-five percent of these specific cells were of the IgA isotype. We have come remarkably close to confirming this number by using a completely different technique and a heterologous rather than a homologous murine model. On the basis of data from Table 2, we have shown that 89% of small intestine LP RRV-specific ASC were secreting IgA. This finding may not be surprising, however, as IgA-secreting cells of all specificities constitute 88% of ASC in this tissue (Table 1).

Another important point of agreement between the present study and the data reported by Dharakul et al. is that approximately half of the cells bearing stainable immunoglobulins were virus specific in their study. This correlates well to our finding of almost 50% of ASC in the LP being virus specific at 20 to 60 days postinfection.

A similar predominance of local IgA response has also been demonstrated with other intestinal antigens such as the cholera toxin, and a correlation with protection has been demonstrated (30, 58). The local secretion of IgA is therefore now considered to be the important effector mechanism of mucosal immunity. It has been estimated that more than 40 mg/kg of body weight of IgA is secreted into the gut lumen daily, which is more than the total production of IgG, the dominant immunoglobulin in serum (9). The generation of these large amounts of secretory antibodies is dependent on the large number of IgA-producing cells in the LP, estimated to account for 70 to 80% of all immunoglobulin-producing cells in humans as well as mice (61, 62).

The ELISPOT technique developed for quantitating intestinal ASC in a precise and efficient manner now offers the means to study more effectively the response to rotavirus in this important organ of immunity. The detection of rotavirus-specific ASC in the LP as early as 4 days postinfection (compared with 10 days by immunohistochemical techniques) indicates the improved sensitivity of the ELISPOT in detection of smaller specific responses.

The overall sensitivity of this system for the identification of ASC in the small intestinal LP can be compared with results in published articles that reported the background production of immunoglobulin in the mouse small intestine as a function of age (62). A rapid rise was seen in small intestinal IgA ASC beginning at 3 weeks of age and reaching a plateau of about $10^7$ IgA ASC by 6 weeks of age. Mice in this study designated 20 days postinfection are approximately 4 to 5 weeks of age, and other groups range up to about 10 weeks. In our study, IgA ASC were not significantly different in any of the age groups beyond 20 days postinfection, with the average IgA ASC in LP being about 150,000 per $10^6$ mononuclear cells. For comparison with the results of Van der Heijden et al., who reported $10^7$ ASC from $10^6$ LP mononuclear cells, our rate of 150,000 per $10^6$ mononuclear cells is about $1.5 \times 10^5$ per animal, which is virtually the same result. The fact that we observed total IgA levels at mature levels in mice by the time they were 3 weeks of age suggests that rotavirus infection hastened normal ontogeny. A single experiment on naive mice 14 days of age showed smaller number of IgA ASC in the LP compared with that in infected mice, but a larger number of uninfected controls at ages less than 3 to 4 weeks would be required to directly address this problem.

The incidence of surface and internal T- and B-cell markers in the lymphocyte populations of spleen and the different gut-associated lymphoid tissues of mice have been described and reviewed previously (6, 24, 33, 41, 42, 59, 60). Through the ratio of T to B cells varies somewhat depending on the technique used, the reported differences between these tissues do not account for the marked differences we report.

### TABLE 2. RRV-specific ASC isolated from indicated tissues between 20 and 60 days after RRV infection

<table>
<thead>
<tr>
<th>Tissue</th>
<th>IgM ASC ± SEM</th>
<th>IgG ASC ± SEM</th>
<th>IgA ASC ± SEM</th>
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<tbody>
<tr>
<td>Spleen</td>
<td>39 ± 7</td>
<td>58 ± 13</td>
<td>54 ± 18</td>
</tr>
<tr>
<td>Peyer's patches</td>
<td>370 ± 121</td>
<td>774 ± 158</td>
<td>2,600 ± 321</td>
</tr>
<tr>
<td>Mesenteric nodes</td>
<td>7 ± 2</td>
<td>29 ± 9</td>
<td>38 ± 11</td>
</tr>
<tr>
<td>Lamina propria</td>
<td>69 ± 30</td>
<td>8,483 ± 2,849</td>
<td>68,183 ± 8,530</td>
</tr>
</tbody>
</table>

*Results are expressed as the number of ASC per $10^6$ mononuclear cells ± SEM (n = 29).
in the number of ASC. The use of the total number of viable mononuclear cells as the denominator is a convention used by most authors studying the immune response in terms of ASC. This approach circumvents the discrepancies involved in identifying T and B cells by the use of different tests and attempting to distinguish immunoglobulin-containing plasma cells and membrane immunoglobulin-bearing B cells which may or may not be secreting antibodies. Moreover, since the total number of mononuclear cells in spleen and LP is known to be in the order of $10^8$ per mouse and in the order of $10^7$ in the PP and MLN, the relative contribution of each organ to the total body response can be appreciated (64).

Although the location and isotype of the intestinal antibody response to rotavirus reported herein is similar to the responses to other intestinal antigens that have been previously described, the overall magnitude of the response to rotavirus is very strong compared with those to other antigens that have been studied with similar techniques. For instance, ASC specific for cholera toxin were quantitated in the LP by ELISPOT using petri dishes as the solid phase, and only 23 ASC per $10^6$ mononuclear cells were found to be secreted after a single oral dose of 10 $\mu$g of cholera toxin, which could be enhanced to 7.8% of the total active ASC in this version of the ELISPOT assay, whereas the overall RRV-specific response in the LP in our study was 48%. Keyhole limpet hemocyanin, which alone does not stimulate a response when orally administered to mice, can be rendered antigenic if administered with cholera toxin. When the ELISPOT assay was used to quantify this response, peak numbers of small intestinal LP lymphocytes of between 1,000 to 10,000 ASC per $10^6$ mononuclear cells were noted, but no information regarding the total ASC present was available (69).

The response to another orally administered (but nonreplicating) viral antigen, the formalin-inactivated influenza virus, was studied by using the ELISPOT technique (7). LP response was not studied, but the virus-specific ASC in PP cells were quantitated. The peak response was among PP-derived IgA-secreting cells and was 27 ± 1.6 ASC per $10^6$ mononuclear cells for virus alone and 326 ± 1.5 ASC per $10^6$ mononuclear cells when virus was administered with cholera toxin and after PP cells had been cultured in vitro for 2 days in the presence of viral antigen. These levels are markedly lower than we report for the same tissue and isotype (RRV-specific IgA ASC in PP = 2,600 ± 321) without either adjuvant or in vitro culture with antigen. These comparisons dramatically demonstrate the power of replicating rotavirus as a mucosal antigen. Though the other studies have used petri dishes as the solid phase (which, in our experience, may result in an underestimate of the total number of spots), the exponential differences in the small intestinal LP responses could not be attributed to such technical differences, especially considering that the responses in the other tissues are comparable.

The responses to rotavirus in the MLN and the spleen are more similar to that described for other antigens. When the response to the parasite Trichinella spiralis was quantitated in rat MLN, ASC were found to peak at 751 ± 298 IgA ASC per $10^6$ mononuclear cells, with a peak at 12 days postinfection (65), but IgG and IgM responses were not studied. The comparable RRV-specific IgA response in MLN at 10 days postinfection was 91 ± 37 ASC per $10^6$ mononuclear cells, though IgG and IgM responses were the dominant at early time points (601 ± 300). Splenic lymphocytes have generally been studied for IgM or IgG after parenteral immunization with agents such as Streptococcus pneumoniae polysaccharide or Streptococcus mutans, leading to 150 to 500 ASC per $10^6$ mononuclear cells (47, 66).

The circulating and intestinal virus-specific immunoglobulins after a homologous infection in seronegative mice have been described by Sheridan et al. (53). They demonstrated an early IgM response in serum, followed by the appearance of IgA and IgG in serum and a predominant IgA response in intestinal washings starting at days and continuing to rise through their period of study (21 days). In our study, specific antibody titers for IgA and IgG in serum and IgG in feces continued to rise to 20 days postinfection and persisted at high levels, with some downward trend at 60 days postinfection (IgM titers were not studied). The peak serum IgG occurred at a later time, possibly reflecting the continued accumulation of IgG in the serum due to the longer half-life of this immunoglobulin class. This delayed peak of serum IgG has previously been described in studies of human volunteers (2).

Mice are susceptible to rotavirus illness only between 1 to 13 days of age and are therefore generally considered unsuitable for use in studying the determinants of active disease (69). Adaptation of precise measures of antibody responses, such as the ELISPOT assay, may prove useful in attempts to determine the antibody correlates of disease protection.

The effects of virus dose and virus strain in the murine model of rotavirus infection have also been well studied. RRV has been shown to replicate in the intestinal epithelium and cause severe disease. However, only at very low doses of virus does replication occur to levels above the inoculated dose (43). In this study, a relatively large dose of rotavirus was administered, approximately 1 log unit more than the dose needed to cause disease in 50% of the pups (35), and infection therefore resulted in grossly evident diarrhea in more than 95% of the pups. This large dose results in the presence of considerable amounts of both replicating and nonreplicating antigen available in the intestine for presentation to the intestinal immune system. The relative contribution of each and relative importance of epithelial or the M-cell pathway taken by the replicating and nonreplicating antigen remain to be elucidated.

This model allows for the study of the local antibody-secreting cells and can be modified to study not only the virus-specific response but also protein- or epitope-specific responses. Recent developments in biotechnology have enhanced the enthusiasm for efforts at immunoprophylaxis by vaccination with synthetic viral peptides, bacterial and viral vectors, and reassortant strains. The effectiveness of these agents depends on the determination and optimization of their immunogenic properties. We believe that this model for studying local immune response will help realize the potential of these approaches.

REFERENCES


