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VP4-Specific Intestinal Antibody Response to Rotavirus in a Murine Model of Heterotypic Infection

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We have adapted a murine model of heterotypic rotavirus infection for the purpose of evaluating the intestinal antibody response to an infection that mimics human vaccination. Neonatal mice were infected with the rhesus rotavirus (RRV). The enzyme-linked immunospot assay was used in order to avoid common artifacts in the quantitation of intestinal immune responses inherent in measurements of luminal or serum immunoglobulins and to obtain easily quantifiable data in a flexible and convenient format. Functionally active lymphocytes were harvested from the spleen, small intestinal lamina propria, Peyer's patches, and mesenteric lymph nodes and processed into single-cell suspensions. Antibody-secreting cells (ASC) were quantitated from 5 to 50 days after infection for total, RRV-specific, baculovirus-expressed VP4-specific, and single-shell RRV-specific ASC secreting either immunoglobulin G (IgG), IgM, or IgA. The response to VP4 constituted less than 1.5% of the total virus-specific response, which was located almost exclusively in the gut and was 90% IgA. Intestinal ASC were directed overwhelmingly toward proteins incorporated in the single-shell particle, predominantly VP2 and VP6. We conclude that the antibody response to VP4, thought to be the site of the important neutralization sites conserved among several rotavirus serotypes, is an extremely small portion of the overall antibody response in the intestinal tract.

Rotavirus has been clearly shown to be the major cause of severe gastroenteritis and a leading cause of death in infants and young children around the world. Efforts at vaccine development have been proceeding rapidly, but difficulty has emerged in obtaining sufficiently broad protection against disease caused by the several human rotavirus serotypes. One of the barriers to a highly successful vaccine strategy has been the lack of a basic understanding of the factors that determine many aspects of the immune response to vaccination.

The principal failure of vaccine candidates in field trials may have been due to the limited immune response elicited to regions of the rotavirus that mediate heterotypic neutralization. This characteristic, as presently understood, is mediated by antibodies directed at limited regions of the two outer shell proteins: the VP5 segment of VP4, and specific regions of VP7 (34, 37, 62, 63). Antibodies directed at numerous other epitopes on these two proteins mediate strain- or type-specific neutralization. Considerable confusion has arisen concerning the relative antigenicities of these two proteins and among the various homotypic and heterotypic neutralization regions within them. The mechanisms by which particular regions of these proteins are targeted by the antibody response are obviously among those of critical importance in determining the effectiveness of vaccination (22).

We have developed a model of murine intestinal antibody response to heterologous infection with rhesus rotavirus, a candidate vaccine strain used extensively in field trials (11, 18, 19, 53, 54, 67). This model, which uses the enzyme-linked immunospot (ELISPOT) assay to enumerate specific antibody-secreting cells (ASC), has been characterized previously in terms of the time course of the response, tissue localization, and isotype distributions (40a).

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This model of immune response has several advantages over other approaches that measure titers of antibodies in serum or intestinal contents or in vitro culture systems, as the results are not confounded by antibody degradation, mucus entrapment, tissue culture artifacts, or other well-known deficiencies of these measurements.

In this article, we report the VP4-specific response of intestinal lymphocytes following a single enteral rhesus rotavirus (RRV) infection. This approach has been made possible by the expression of antigenically authentic VP4 from baculovirus expression systems, which provides sufficient VP4 to serve as an antigenic target in the ELISPOT assay (34, 36, 37). Previous attempts to study protein-specific responses have been limited by the lack of adequate quantities of isolated rotavirus proteins of antigenic similarity to assembled capsid proteins. As an alternative approach, an epitope-specific blocking immunoassay with monoclonal antibodies has been used to indirectly assess the antibody response to particular rotavirus proteins and specific epitopes contained by them (22, 56). The production of antigenically conserved rotavirus proteins in the baculovirus expression system, however, promises to permit comprehensive determinations of the protein specificity of antirotavirus antibody responses in a variety of immunoassays. The ability of this system to detect intestinal responses to VP4 with excellent sensitivity and specificity will allow the development of a greater understanding of the mechanisms that determine the immunogenicity of a particular protein component of a complex replicating intestinal antigen and may lead to improved vaccine strategy.

MATERIALS AND METHODS

Animals and immunizations. Pregnant $CD2F_1$ 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 experi-

ment. Serum samples 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°C (30). The dose of virus administered by gastric intubation was 10^7 PFU administered in 100 μl of tissue culture supernatant. The virus was not deliberately trypsin-activated in vitro prior to administration. Pups were restricted from suckling for 45 min before and after immunization. This dose of virus resulted in diarrhea in greater than 95% of the pups, usually seen between days 2 and 4 after infection. Two littermates were sacrificed at defined time intervals, and the spleen, mesenteric lymph nodes, small intestine, intestinal contents, and serum were obtained. Experiments included pups of both sexes whenever possible to minimize the significance of sex-specific influences on the immune response, if any.

Lymphocyte isolation procedure. Small intestine contents were removed as described above, and the intestine was collated over a narrow spatula. Macroscopically visible Peyer's patches were dissected. Lamina propria lymphocyte isolation was accomplished by a modification of the procedure first described by Davies and Parrott and modified by Van der Heijden et al. with EDTA and collagenase, which allows recovery of functionally active intestinal lymphoid cells (66). The cell suspension was subjected to a discontinuous Percoll gradient centrifugation to isolate viable mononuclear cells, which were enumerated by trypan blue exclusion. Spleen, Peyer's patches, and mesenteric lymph node 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.

Expression of VP4. VP4 was expressed by a recombinant baculovirus vector in Sf9 cells as previously described (37). The hemagglutination titer of the sonicated preparation was 1:2,000. This preparation was diluted 1:50 in Tris-saline-calcium chloride (TNC) buffer before use. The cell extracts were sonicated for 20 s and used as the capture antigen in the ELISPOT assay.

ELISPOT. We devised a modification of the ELISPOT technique to measure the number of ASC present in spleen, lamina propria, Peyer's patches, and mesenteric lymph nodes (14). We individually measured immunoglobulin G (IgG)-, IgM-, and IgA-secreting cells in each of the tissues. We measured all cells secreting antibody specifically directed at double-shelled RRV, single-shelled RRV, or baculovirus-expressed VP4. Immobilon PVDF (Millipore), a white hydrophobic polyvinylidene difluoride membrane, was used as the protein-binding matrix. The membrane was mounted in a 16- or 28-lane Miniblotter (Immunetics, Cambridge, Mass.).

The membrane was coated with the desired capture antigens, which included rotavirus elements listed above diluted in TNC or rabbit anti-mouse IgG, goat anti-mouse IgM, or goat anti-mouse IgA diluted in phosphate-buffered saline (PBS). The optimal concentrations for the antigen capture substances had been determined previously in titration experiments with appropriate hybridoma cells as positive and negative controls. Rotavirus used as capture antigen was purified on cesium chloride density gradients as previously described (58). Single-shelled particles, devoid of the VP4 and VP7 proteins constituting the outer shell, were produced from double-shelled particles by EDTA treatment as described previously (16).

TABLE 1. Viral protein specificity of hybridoma cells secreting RRV-specific monoclonal antibodies in the ELISPOT assay

Antibody	Specificity	RRV/IgG[a]	VP4/RRV[b]	SS/DS RRV[c]
7A12	VP4	0.7 ± 0.1	1.0 ± 0.1	0.0
4F8	VP7	1.0 ± 0.036	0.0027 ± 0.0019	0.0
255/60	VP6	0.7 ± 0.038	0.0	0.9 ± 0.062

[a] Ratio of spots generated by identical numbers of hybridoma cells in RRV and IgG capture lanes. This illustrates the integrity of the rotavirus particles used as the capture antigen.

[b] Ratio of spots generated by identical numbers of hybridoma cells in VP4 and RRV capture lanes. The VP4 from the recombinant baculovirus expression system was present in adequate amount to permit antibody 7A12 to give approximately as many spots as with intact rotavirus particles, and the specificity was very high, as there was no cross-reactivity with VP6- or VP7-specific hybridomas.

[c] Ratio of spots generated against EDTA-treated single-shelled (SS) RRV, which was then rebanded in an isopycnic gradient, compared with the spots generated against intact, gradient-purified double-shelled (DS) RRV. Single-capsid virus is predominantly VP6 (65%) and VP2 (19%) (17).

This template was then incubated at 37°C for 2 h on a rocking platform. Lanes were washed with PBS to remove excess capture antigen, and then the membrane was blocked with PBS containing 5% fetal bovine serum and incubated for 1 h. Cell viability was assessed by trypan blue exclusion, and the appropriate dilutions in RPMI 1640 medium containing 2% fetal bovine serum were made so that a countable number of spots resulted in each lane after 60 μl (28-lane template) or 140 μl (16-lane template) of cell suspension was incubated on the capture antigen for 2 h at 37°C. Once the incubation was complete, the cells were washed from the membrane with PBS. Detection of bound mouse immunoglobulin was accomplished by an isotype-specific biotin-avidin-horseradish peroxidase detection system (Vector Labs, Burlingame, Calif.) with the precipitating substrate aminoethylcarbazole (A5754; Sigma Chemical Co., St. Louis, Mo.), which results in red-brown spots. The spots were enumerated under magnification with an Olympus SZ-PT stereo microscope with a halogen fiberoptic light source.

The number of spots was expressed in terms of ASC per 10^6 viable mononuclear cells. Within experiments, multiple lanes of a particular type were combined to determine the total number of spots for that number of cells. This result was the normalized to spots per 10^6 mononuclear cells. Comparisons among means of groups were done by analysis of variance and multiple comparisons were done by Fisher PSLD and the Scheffe *F* test or by *t* tests with the StatView II program (Abacus Concepts, Berkeley, Calif.) on a Macintosh computer.

RESULTS

ELISPOT. The specificity of the ELISPOT assay was established by the use of rotavirus-specific hybridomas. Integrity of rotavirus antigen was monitored by hybridomas 4F8 (VP7 specific), 7A12 (VP4 specific), and 255/60 (VP6 specific) (34, 37, 55). All hybridomas secreted IgG monoclonal antibodies. Table 1 presents the results of control experiments in which the hybridoma panel was tested in ELISPOT against intact double-capsid rotavirus particles, EDTA-treated single-capsid virus, recombinant baculovirus-expressed VP4, and anti-mouse IgG. The ratio of RRV-specific to total IgG-secreting hybridomas detected by the ELISPOT assay was between 0.7 and 1.0 for both the inner

TABLE 2. Response of double-shelled RRV-specific and VP4-specific ASC harvested 10 to 50 days after RRV infection

ASC specificity	Mean no. of ASC/10^6 mononuclear cells ± SEM (n = 19)			
	Lamina propria	Spleen[a]	Peyer's patches	Mesenteric lymph nodes
Total				
IgG	22,579 ± 4,191	2,229 ± 13	2,759 ± 337	405 ± 54
IgM	91 ± 26	2,318 ± 14	507 ± 73	92 ± 10
IgA	157,699 ± 14,206	174 ± 18	4,851 ± 394	371 ± 62
Double-shelled RRV				
IgG	10,014 ± 3,239	56 ± 9	952 ± 212	31 ± 9
IgM	1,305 ± 1,008	37 ± 7	278 ± 151	7 ± 3
IgA	78,683 ± 8,377	41 ± 11	3,076 ± 332	41 ± 12
VP4				
IgG	388 ± 132	44 ± 7	27 ± 8	6 ± 2
IgM	123 ± 72	49 ± 9	20 ± 7	4 ± 2
IgA	837 ± 374	5 ± 2	42 ± 12	9 ± 3

[a] Spleen IgG- and IgM-secreting ASC consistently contained ASC that secreted antibodies directed against the fetal calf serum-blocking proteins and the viral antigen. This background was not a function of the age or virus exposure status of the mice. The average value for spleen IgG was 11 ASC/10^6 cells, 2 standard deviations above that was 25 ASC/10^6 cells; for spleen IgM, the average was 24 ASC/10^6 cells, and 2 standard deviations above that was 50 ASC/10^6 cells. These values are suggested as cutoffs above which one is likely to be observing the actual virus-specific response. The splenic responses are small in any case, and more so if these values are subtracted. The data as displayed have not been adjusted to account for this effect.

shell- and outer shell-specific hybridomas, indicating that intact viral particles were functioning efficiently as antigenic targets for secreted antibodies. The antigenic integrity of the expressed VP4 was confirmed by the pattern of reactivity of hybridoma 7A12, which produced as many spots when VP4 was used as a capture antigen as when whole virus was used, while the VP4 preparation did not capture antibodies secreted by the other hybridomas. Verification that the assay measured secreted antibodies and not membrane-bound fragments was provided by experiments in which hybridoma cells were incubated in cycloheximide prior to the ELISPOT assay. In these experiments, the number of ASC detected declined 70 to 90% (data not shown), a typical result reported by others for this technique (13).

Time course of ASC and RRV-specific ASC appearance. Cells harvested from spleen, Peyer's patches, mesenteric lymph nodes, and small intestine lamina propria were harvested from 3 to 60 days after infection with RRV. These cells were independently assayed for secretion of IgG, IgM, and IgA antibodies as well as those antibodies specific for gradient-purified double-shelled rotavirus particles, baculovirus-expressed VP4, and, in some experiments, those specific for single-shelled RRV.

The overall response to the virus was concentrated in IgG and IgA in the small intestine lamina propria, as Table 2 illustrates and is discussed below. For this reason, data illustrating the time course of the response will be presented for this tissue and these antibodies only.

Figure 1 illustrates the time course of the development of total IgG and IgA ASC in the lamina propria of the small intestine of animals that were infected with RRV at 9 to 12 days of age. In addition, IgG- and IgA-secreting cells specific for RRV or VP4 are also represented. The marked rise in IgA ASC between days 15 and 30 of age (approximately days 5 and 20 postinfection) is consistent with previous studies on murine intestinal IgA ontogeny (see Discussion) (1, 65). The

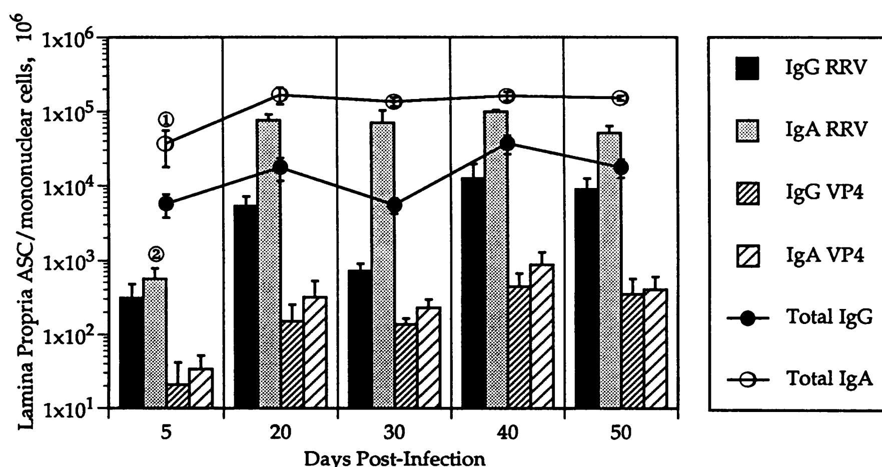

FIG. 1. Immunoglobulin-secreting ASC in lamina propria. Lymphocytes were harvested from the murine small intestinal lamina propria between 5 and 50 days after infection with RRV and assayed for IgG or IgA ASC in the ELISPOT assay. The results are expressed in terms of ASC per 10^6 viable mononuclear cells ± SEM. Antigenic targets used in these assays included affinity-purified goat anti-mouse IgG or IgA, intact purified RRV, and recombinant baculovirus-expressed VP4. Statistical comparison of groups was done by analysis of variance, with the Scheffe F test used for multiple comparisons where appropriate. Significance at 95% is indicated by the following symbols: 1, total IgA on day 5 was significantly lower than at any other time; 2, RRV-specific IgA was significantly lower on day 5 than on day 20, 30, or 40 but not on day 50. In all other cases, differences among time points are not significant at the 95% confidence level.

TABLE 3. Response of double-shelled and single-shelled RRV-specific ASC harvested 10 to 50 days after RRV infection[a]

ASC specificity	Mean no. of ASC/10^6 mononuclear cells ± SEM (n = 12)			
	Lamina propria	Spleen[b]	Peyer's patches	Mesenteric lymph nodes
Double-shelled RRV				
IgG	13,608 ± 7,763	57 ± 12	423 ± 192	27 ± 14
IgM	21 ± 14	55 ± 13	13 ± 6	6 ± 3
IgA	81,767 ± 13,555	23 ± 5	1,954 ± 530	32 ± 17
Single-shelled RRV				
IgG	13,364 ± 7,768	32 ± 6	387 ± 135	25 ± 12
IgM	271 ± 196	33 ± 7	11 ± 4	5 ± 1
IgA	86,437 ± 18,594	16 ± 5	1,883 ± 303	27 ± 11

[a] Data are analagous to those presented in Table 2, except that the 12 experiments in which paired observations of both single- and double-shelled RRV-specific ASC were recorded have been excerpted for direct comparison.

[b] Spleen IgG- and IgM-secreting ASC are displayed without correction for the background discussed in Table 2, footnote *a*. To correct for this, 2 standard deviations above the average background can be subtracted; these values are 25 ASC/10^6 cells for spleen IgG and 50 ASC/10^6 cells for spleen IgM. No background was detected in other tissues or with other antibody types.

development of a plateau at this time is relatively early, however, and the rate of development of the total pool of IgA ASC may have been enhanced by the RRV infection, although that conclusion cannot be drawn from these data alone.

The RRV-specific antibody responses of the small intestine lamina propria lymphocytes were followed through 50 days postinfection. The very first small intestine ASC responding to rotavirus were noted on day 3 to 4 postinfection but rose rapidly to a plateau which was established between days 20 and 30 postinfection. It is very clear that the response is almost entirely IgA. While it appears from the data presented that the IgA response to RRV trends downward between 40 and 50 days after infection, the limited data that we have obtained for animals at 90 and 180 days postinfection suggest that this decline is not significant at these times. At all time points from day 20 onward, there was very little difference in magnitudes of responses or the relative contributions of IgG, IgM, or IgA antibodies. For this reason, data from these time points were pooled for further analyses.

The time course of the small intestine antibody response to the RRV outer shell hemagglutinin VP4 parallels that of the response to whole virus. The overall level of the VP4-specific response was less than 2% of the response to whole virus at all time points (note logarithmic axis of Fig. 1) except day 5 postinfection, but this was not statistically significant (Fig. 1).

VP4 specific ASC for four tissues during days 20 to 60 postinfection. The frequency of ASC in the small intestine far exceeded that in the other tissues studied (Table 2). The distribution of the rotavirus-specific ASC mirrors the distribution of all ASC. Roughly half of all small intestinal ASC were virus specific. This finding is consistent with other data we have reported (40a). The small intestinal lamina propria contained the greatest frequency of VP4-specific ASC of the tissues studied. The other tissues had far fewer relative and absolute numbers of VP4-specific ASC, but in the case of spleen and mesenteric lymph nodes, there were significantly more VP4-specific ASC in the RRV-specific population than one would expect from an extrapolation of the lamina propria frequencies. However, the small number of specific ASC in these tissues makes analysis of ratios difficult. In the case of the spleen, the small numbers of background ASC make this ratio meaningless. No such background was observed in the mesenteric nodes; the number of specific ASC was very consistent, and the difference in the ratio compared with that in the lamina propria was very large. The lamina propria ratio was 0.012 ± 0.004, and that of the mesenteric lymph nodes was 0.356 ± 0.13. Analysis of variance for the tissues showed highly significant differences [$F(3,52) = 15.392$, $P = 0.0001$], and the Scheffe F test for multiple comparisons showed significance at 95% for the difference between the ratio in the lamina propria or Peyer's patches and that in the mesenteric lymph nodes. This finding suggests that variations in viral antigen specificity exist in ASC within different tissues.

Single-shelled and double-shelled RRV. Isopycnic gradient-purified RRV was treated with EDTA to remove the outer capsid containing VP4 and VP7. As shown in Table 1, this single capsid (or single-shelled) preparation demonstrated the desired antigenic characteristics: it was not recognized by hybridomas secreting monoclonal antibodies specific for VP7 or VP4, but it was efficiently recognized by the hybridoma specific for VP6 (255/60). Including all tissues (small intestinal lamina propria, spleen, Peyer's patch, and mesenteric lymph node) and antibody types (IgG, IgM, and IgA) from experiments in which both single-shelled and double-shelled RRV were evaluated for the same pool of cells, single-shelled RRV identified 96.4% ± 0.128% (n = 119) of the ASC identified by intact double-shelled RRV (data not shown). Single-shell-specific ASC results were compared with double-shell-specific ASC results, and the results were studied in an analysis of variance with tissue of origin and antibody type as the variables. No significant differences ($P < 0.05$) or trends ($P < 0.10$) were evident. If all ratios in which either the single-shell-specific ASC or the double-shell-specific ASC result was <100 were discarded (to eliminate the potentially disruptive effects of small spot numbers on the ratios), the results were the same: 97.3% as many single-shell-specific ASC were identified as double-shell-specific ASC overall, and the analysis of variance identified no differences within groups defined by tissue of origin or antibody type. The overall responses to single-shelled and double-shelled RRV were not significantly different (Table 3).

Early response, days 3 to 7 postinfection. Four litters studied at days 3, 5, and 7 postinfection have been grouped as day 5. This early-response profile is notable for, in general, a greater contribution of IgM and also IgG to the ASC response than was seen at day 20 and onward. The activity in the mesenteric lymph nodes was remarkably high at these early times, and in fact the greatest frequency of virus-specific ASC of all types occurred at this time, although the absolute number of cells in this tissue was relatively small (about 10^5 per animal). VP4 specificity at

these early time points may also be somewhat different than during the mature response, although only lamina propria demonstrated a significant difference in the total VP4-specific/RRV-specific ratio at day 5 versus days 20 to 50. The day 5 ratio was 0.3 ± 0.2, while the ratio for the mature response was only 0.0093 ± 0.0026 ($P = 0.0093$ by two-tailed t test).

DISCUSSION

We have quantitatively characterized the intestinal antibody response to a heterologous infection with a simian rotavirus in a murine model. This virus is currently under investigation in field trials as a human vaccine strain, using the Jennerian model of a naturally attenuated animal virus which nevertheless retains significant antigenic similarity to pathogenic human strains (31). Many aspects of the host response to viral infection of the intestinal tract are as yet unknown, but this information is obviously of interest in the development of vaccine strategies and can assist in the improvement of our understanding of basic mechanisms of intestinal immune defenses. The present study addresses particularly the specificity of the intestinal antibody response for the viral surface protein VP4.

The selection of a homologous or heterologous animal model may be an important determinant of several aspects of the intestinal antibody response to rotavirus infection or vaccination. Work in murine models has clearly demonstrated that heterologous infection (i.e., a laboratory infection of mice with a virus that naturally infects another species) is a far less efficient process than is homologous infection with a murine rotavirus. The "natural" homologous infection produces disease with a very small input of infectious virus, far greater viral replication occurs, and the infection is more readily spread to others (23, 27, 52, 59, 73). Limited replication of RRV does occur in the mouse intestine (52), however, and a very large immune response is elicited. It is as yet unclear whether the higher degree of viral replication that occurs during a homologous infection is a significant determinant of the antibody response. Presently available evidence does not clearly show the importance of this factor in the murine model, but investigations are not complete. Dharakul et al. provided direct characterization of the intestinal response to homologous murine infection and found a time course and magnitude of response which were expressed in terms of antibody-bearing cells identified in immunohistochemical studies of intestinal tissue sections (15). These studies documented that 50% of immunoglobulin-bearing cells were virus specific, and the peak response was not seen until beyond 30 days postinfection. These findings bear remarkable similarity to our data from a heterologous model (Tables 2 and 3). The protein specificity of antibodies elicited in response to homologous or heterologous infection may also be similar. Taniguchi et al. have recently reported human studies comparing the response to natural infection with that to vaccination with simian RRV with the epitope-specific blocking immunoassay. The authors' conclusion was that the immunity patterns found after natural infection and vaccination "seem to be essentially the same" (64). The heterologous model studied here (murine infection by a simian serotype 3 virus) has been widely used to evaluate serological markers and cytotoxic T-cell responses to rotavirus infection (42–46, 48) and is a logical choice to use to study the response to a process similar to human vaccination with naturally attenuated animal viruses.

Rotaviruses contain two outer shell proteins, VP4 and VP7, that elicit neutralizing antibodies, and which surround an inner protein capsid constructed mostly of VP6 and VP2 (17, 38). The specificity of this rotavirus ELISPOT assay is elegantly controlled by the use of specific monoclonal antibodies directed at the two outer capsid proteins and also VP6. Antibody 7A12 was used as a VP4-specific control, and this antibody demonstrated VP4-specific activity at a rate of 88% of RRV-specific activity. Antibody 4F8, directed at VP7 (a capture antigen not present in the expressed VP4 preparation but easily detected on whole RRV), was very efficient at detecting virus but virtually never resulted in false-positive spots in VP4 capture zones. Antibody 255/60, directed at the inner-shell protein VP6, also detected whole RRV but never VP4. Therefore, as used to detect ASC in this assay, our capture antigens for RRV and VP4 provided comparable sensitivity and excellent specificity. We performed large numbers of experiments with nonimmunized and mock-infected mice and proved that the virus-specific responses in these animals were negligible, with the exception of spleen cells secreting IgG or IgM (but not IgA), which consistently produced 10 to 50 ASC/10^6 mononuclear cells to capture zones with double- or single-shelled RRV or expressed VP4. Approximately one-third of this response was directed at lanes with no capture antigen that were blocked with fetal calf serum. These spots were present at the same rate regardless of the age of the mice and occurred in mice with no serological antirotavirus activity. We assume that these cells represent activity unrelated to virus infection.

Passive protection studies in mice and several other species have confirmed the ability of neutralizing antibodies directed at either outer capsid protein to protect against disease (2, 5, 17, 38, 39, 46–48). Initially, studies in hyperimmunized animals indicated that VP7 was the primary target of serum neutralizing antibodies, as illustrated by the apparent segregation of serotype classification with VP7 in gene-coding analyses with temperature-sensitive mutants, although characterization of monoclonal antibodies revealed neutralization sites on what is now designated VP4 (24, 28, 29). While the contributions of the gene 4 and gene 9 products (VP4 and VP7, respectively) mediating viral neutralization were further clarified in genetic studies with simian and bovine rotavirus reassortants, data concerning the relative immunogenicity of the two proteins during infection or vaccination and the roles of antibodies to these proteins in active disease protection remain confusing (22, 45). It has long been recognized that regardless of the route of antigen presentation, the major inner capsid protein VP6 (which does not mediate neutralization or protection) elicits the greatest antibody response of all the rotavirus proteins (17).

VP7 was initially thought to be immunodominant among the neutralizing antibodies from studies of hyperimmune sera obtained from rabbits that had been hyperimmunized with simian rotaviruses (8, 12, 32). Several subsequent studies with a variety of techniques identified important and sometimes dominant roles for neutralizing antibodies to VP4 elicited by parenteral inoculation or intestinal infection in serum and intestinal secretions of mice and piglets (25, 26, 45, 47). There was some evidence that the oral route of antigen presentation in mice may influence the specificity of the antibody response toward VP4 (57). Children were found to mount an antibody response to VP4 after oral inoculation with two candidate vaccine strains or in some cases of natural infections, but in other studies children seemed to have a lesser heterotypic response than adults (6, 22, 56, 61). Also, adults orally challenged with a virulent human rotavirus had a dominant neutralizing antibody response directed at VP4. Other studies, however, have continued to demon-

strate the dominance of VP7 as a neutralization antigen after oral presentation of homotypic and heterotypic challenges (69).

The data presented in this article are an attempt to enhance the quantitative information available on the protein specificity of the antibody response in a murine model of heterotypic infection. The ability to obtain this information derives from the adaptation of the ELISPOT assay as a superior tool for quantification of this response and the availability of adequate quantities of baculovirus-expressed, antigenically conserved VP4. Therefore, the relationship of the expressed VP4 to that present on intact rotavirus particles is obviously important in the interpretation of these results. The similarity of these two forms of VP4 has been well established in prior publications (36, 37, 68). Expressed VP4 has been shown to share the functional characteristics of native VP4 as a hemagglutinin. Immunization of mice with expressed VP4 resulted in the production of high-titer rotavirus antiserum, which included predicted serotypic cross-reactivities based on previously characterized regions located on the VP5 fragment of VP4. Furthermore, a highly characterized library of 11 monoclonal antibodies directed at six different antigenic domains of VP4 (including the antibody used in this study, 7A12) were all demonstrated to bind expressed VP4 nearly as well as or better than whole virus in solid-phase immunoassays (35). There is no evidence to date to indicate any antigenic difference between the expressed VP4 and the VP4 in viral capsids. The antigenic similarity of these two forms of VP4 has led others to attempt to define serologic classifications of rotaviruses based on patterns of antibody reactivity to baculovirus-expressed VP4 (21). Therefore, the data we present, determined from the number of cells responding to expressed VP4 (Table 2), very likely represent a reasonable approximation of the sum of the antibodies directed at native VP4.

The data presented in this article confirm prior studies indicating that VP4 stimulates the production of specific antibodies following heterologous rotavirus infection, and our studies further localize the response to the intestinal mucosa. However, while the overall response to whole virus demonstrated the remarkable potency of rotavirus as an intestinal antigen, VP4-specific ASC constituted less than 2% of the virus-specific cells. VP4 constitutes about 1.5% of total virion mass (17, 33). VP6 and VP2 together constitute 96% of the single capsid particle and were probably the target for a substantial percentage of the ASC in this study. The responses, therefore, seem to be roughly consistent with the relative amounts of each protein present in the virion. It remains to be seen whether the amount of viral replication or the size of the inoculum influences the VP4 specificity of the antibody response.

While no data are presented in this article regarding the antibody response to the other important outer capsid-neutralizing protein VP7, this is obviously a subject of interest. To date, it has not been possible to obtain an expressed VP7 that meets the stringent criteria for antigenic similarity to native VP7 that has been documented for the expressed VP4 used in this study, so it has not been possible to perform the analysis we have reported for VP4. Based on the larger proportion of viral mass that VP7 represents in comparison to VP4 (30 versus 1.5%), one might predict that this protein would constitute a much larger proportion of the immune response. This finding could be consistent with the data and interpretations that we have presented. For instance, the virus-specific lamina propria IgA measurements in Tables 2 and 3 are presented with standard errors of the mean of 15 to 20% of the experimental value, a function of biological variability in the antibody responses. Therefore, our inability to detect a significant difference between the response to double- and single-capsid particles does not rule out the possibility that there is still a difference that may in fact be large enough to account for a considerable VP7-specific response.

The intestinal antibody response to rotavirus reported herein can be compared with the responses to other intestinal antigens that have been described previously. The primary location of the antibody response in the small intestinal lamina propria and the dominance of IgA are consistent with the response to cholera toxin (20, 60). The overall magnitude of the response to rotavirus is very strong in comparison with that to other antigens that have been studied with similar techniques, which in many cases respond at a level comparable to that to VP4 alone (10, 60, 66, 70). This fact may explain the measurable functional effects of anti-VP4 antibodies in some studies, which may be detectable despite the relatively small fraction of the total antibody response to virus that these antibodies represent. We have not studied the anti-VP4 antibodies in neutralization assays, and we wish to emphasize that even though they represent a minor element of the overall antibody response, these relatively small numbers of VP4-specific antibodies may still represent a significant contribution to the overall neutralizing capacity of this response in light of the fact that antibodies directed to antigens on the single-capsid particles are not neutralizing.

Rotavirus is known to bind to and perhaps replicate within M cells and be transmitted directly to Peyer's patch cells, one possible pathway of presentation to the immune system that is used by many other nonreplicating or mucosally noninvasive antigens (e.g., horseradish peroxidase, cholera toxin, and reovirus) (7, 10, 49, 50, 51, 72). An alternative mechanism for antigen presentation that seems plausible for rotavirus involves infection of villous tip epithelium and an encounter with antigen-presenting cells in the intraepithelial compartment or in the lamina propria, a mechanism which would be suitable for a pathogen such as rotavirus that invades the intestinal epithelium. Also, a role for the intestinal epithelial cell in antigen presentation, now known to have such capability, may also exist (3, 9, 40, 41, 71). While any route of contact with the intestinal immune system probably engenders some antibody response, it is possible that the relative importance of various viral proteins in the response may vary. For instance, viral nonstructural proteins would only be present during viral replication. Epithelial cells may preferentially display certain proteins prominently on the surface with Ia antigens. In short, many factors other than the relative amounts of the viral proteins might be expected to influence the specificity of the antibody response, an issue which we are investigating further with this model.

Assuming that the object of the host response to rotavirus infection is subsequent disease protection that is associated with antibodies directed to outer capsid structures, ELISPOT quantification of the protein-specific antibody response to rotavirus infection in the mouse model illustrates clearly the futility of a great portion of this response when enteral immunization is performed with large doses of heterotypic virus. Future vaccine strategies may benefit from attempts to focus more of the antibody response toward viral neutralization antigens such as VP4.

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