Persistence of Intestinal Antibody Response to Heterologous Rotavirus Infection in a Murine Model beyond 1 Year

ROBERT D. SHAW,* AYUB A. MERCHANT, WILLIAM S. GROENE, AND EDWARD H. CHENG
Department of Medicine, Northport Veterans Affairs 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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We used an ELISPOT (enzyme-linked immunosorbent spot) assay to quantitate the long-term rotavirus-specific intestinal antibody response in a murine model. The frequency of murine intestinal antibody-secreting cells (ASCs) was followed for a period of 1 year after a single dose of rhesus rotavirus (10^6 PFU) was administered at 10 days of age. Some animals were boosted at that time with a second dose. One year after infection, virus-specific ASCs declined from acute-phase levels, but they were still present at significant levels (1.32 × 10^5 virus-specific ASCs per 10^6 intestinal mononuclear cells; approximately 17% of the previously reported response at 1 month after infection). A booster dose 1 year after the primary infection produced a 100% increase in virus-specific ASCs but did not restore the response to that of the primary infection.

Rotavirus is the most important cause of severe gastroenteritis worldwide. 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 understanding the mucosal immune response to replicating viral antigens. The murine model of heterotypic rotavirus infection has several features in common with human disease that have led to the frequent use of this model by investigators who are attempting to define the mechanisms of intestinal immune responses to viral infections. We have previously reported (5, 8) on the use of the ELISPOT (enzyme-linked immunosorbent spot) assay for the quantitation of several characteristics of the antibody response of the murine intestine in this model, including the specificity of the response for the outer capsid neutralization protein VP4. Our previous reports focused on the acute-phase response to infection. In this report, we describe the persistence of the intestinal immune response 1 year after rotavirus infection and the effect of a secondary enteral exposure to infectious virus.

MATERIALS AND METHODS

The initial handling and enteric infection of CD2F_1 hybrid mice was described previously (5). Briefly, seronegative pathogen-free dams (Taconic Farms, Germantown, N.Y.) delivered pups in microisolator facilities. Sixty pups were fed 10^3 PFU of rhesus rotavirus at 10 days of age, which resulted in diarrhea in >95% of pups on day 3 following infection. Fifteen pups were given sham immunizations of tissue culture medium only (designated naive mice). The adult mice used in the secondary infection experiments did not have diarrhea (as expected). Naive and infected mice were housed in the same room, and sentinel naive mice were assayed monthly for rotavirus antibodies by ELISPOT and neutralization assays, which were consistently negative. Sentinel mice were tested quarterly for a panel of pathogens (including epizootic diarrhea virus of infant mice [EDIM]) by Ahmed Biosafe Inc. (Rockville, Md.), and none were found.

Mice were sacrificed 180 and 360 days after the infectious virus was administered, and also 1 and 2 weeks after a booster dose of infectious virus was administered on day 360. Serum and intestinal fluid were collected, and mononuclear cells were harvested from spleen, Peyer's patches, mesenteric lymph nodes, and small intestinal lamina propria. Cell isolation was performed by using collagenase-EDTA, and cell purification was performed on Percoll as described previously (5).

The ELISPOT and enzyme-linked immunosorbent assay (ELISA) methods used in the present study have previously been described in detail (5, 7, 8). For the ELISA we used isopycnic gradient-purified rhesus rotavirus (RRV) as the capture antigen. Murine rotavirus-specific immunoglobulin A (IgA) was detected with biotinylated goat anti-mouse IgA and avidin-hors eradish peroxidase (KPL, Gaithersburg, Md.). The A_{490} generated by the substrate o-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.) was measured on an automated Bio-Tek reader (EL-309; Bio-Tek, Burlington, Vt.).

The ELISPOT assay used Immobilon P (Millipore) as the solid phase, which was mounted in a lucite template (Immunetics, Cambridge, Mass.) with vertical slots that created “lanes.” The lanes were either treated with goat anti-mouse IgG, IgM, or IgA to identify antibody-secreting cells (ASCs) or optimized amounts of RRV to detect virus-specific ASCs. RRV was grown in MA-104 cell monolayer cultures, released from cells by a freeze-thaw cycle, and purified by fluorocarbon extraction and isopycnic centrifugation in cesium chloride (9). The structural integrity of the virus was assessed by hemagglutination and reactivity in the ELISA with a panel of well-characterized monoclonal antibodies (Mabs) specific for the inner capsid VP6 (Mab 25/60) and the outer capsids VP4 (Mab 7A12) and VP7 (Mab 4F8) (6). Detection of bound immunoglobulin was accomplished with the same immunochromatographic reagents used for the ELISA, except that the precipitating peroxidase substrate was aminoethylcarbazole (Sigma). The red-brown spots were counted by using an Olympus SZ-PT stereo microscope under x2 to x6 magnifications.

RESULTS

The acute-phase antibody response to infection that we have reported previously (5) was >90% IgA, and the cells secreting virus-specific antibodies were mostly localized in
the small intestinal lamina propria. The magnitude of the acute-phase response is represented by data derived 45 days postinfection, as shown in Fig. 1. The virus-specific ASCs approached 50% of the total ASCs in the lamina propria at that time. The persistent response at day 360 was similar to that at 45 days postinfection in type and location, but was of smaller magnitude. Figure 1 depicts the RRV-specific ASCs in small intestinal lamina propria (Fig. 1A) and Peyer’s patch (Fig. 1B) during the acute-phase response at 180 days and 1 year following infection. These tissues are used for illustrative purposes because they contain most of the specific ASCs; the spleen and the mesenteric lymph nodes contain only trivial numbers at any time point. There is a gradual but marked decline in the numbers of virus-specific ASCs in both tissues over time.

Adult mice do not exhibit disease after enteral administration of heterologous rotavirus, an outcome attributable to non-immune system factors that are still incompletely defined. We were interested in determining whether adult mice that were enterally immunized as pups could undergo a boost in intestinal antibody secretion upon challenge at a time remote from the primary infection. We therefore administered RRV (10⁶ PFU in bicarbonate-buffered media) by gavage to barrier-maintained mice 360 days following the primary RRV infection and performed ELISPOT assay analyses of the lamina propria, Peyer’s patch, spleen, and mesenteric lymph node tissues at times of 7 to 21 days following the booster dose.

A small but significant increase in RRV-specific IgA-secreting cells following the secondary infection could be demonstrated in both the lamina propria and the Peyer’s patches in comparison with the number of such cells in the similarly aged but sham-boosted cohort (Fig. 2). However, the preponderance of these cells was located in the lamina propria. Despite careful dissection, it is possible that small numbers of lamina propria lymphocytes may have contami-
nated Peyer’s patch preparations and exerted an important effect on the apparent outcome of the study. However, our Peyer’s patch preparations consistently demonstrate different ratios between IgG and IgA and between total ASCs and virus-specific ASCs, so we concluded that the populations were substantially distinct. Because the greatest amount of ASCs was in the lamina propria, the contribution of the Peyer’s patch cells was not quantitatively important.

Serum and intestinal contents from the two groups were also tested in the ELISA for their rotavirus-specific IgA antibody titers (Fig. 3). The virus-specific activity was not different between the two groups (boosted and nonboosted), but significant levels of persistent virus-specific titers were confirmed in both groups. The levels of persistent antibodies in both compartments were not significantly different from the levels detected in mice at 60 days postinfection (data not shown).

**DISCUSSION**

The data presented here demonstrate two aspects of the intestinal antibody response of the murine model to rotavirus infection. The response that is generated following infection in young mice is maintained throughout the adult life of the mouse, although the levels decline with age. This occurs in the absence of reinfection. If reinfection does occur, the intestinal antibody response can be boosted, although the levels after the secondary infection do not approach those achieved after the primary infection (5).

The finding that the ELISA data were not adequate for the identification of the booster effect is not surprising. The effect was quite small, and the ELISPOT assay has several advantages as a quantitative assay, including reduced numbers of artifacts because of sample dilution, mucous trapping, and proteolysis.

The results obtained by rotavirus intestinal infection can be compared with results of similar experiments performed with the potent intestinal antigen cholera toxin (4). In an ELISPOT study of long-term cholera toxin responses in mice, the intestinal IgA levels declined by about 50% in 6 months and were nil at 24 months. Boosting doubled the specific antibody level at 6 months, and at 24 months boosting restored the level to that obtained during the acute phase of infection. The pattern of persistence of the rotavirus response was similar, and a doubling in antibody levels was obtained by boosting at 12 months, but the level was not restored to the level seen after the acute-phase response.

The population of cells from which virus-specific ASCs persistently arise and the mechanisms responsible for the continued production of antibodies are unclear. Activation of antibody-secreting cells from latent memory seems to be inefficient, but this could be due to either a lack of memory cells or a failure of antigen delivery because of insufficient replication or maturational changes in the intestinal mucosa. A possible role of persistent viral antigen within intestinal antigen-presenting cells beyond 20 days after infection has not been evaluated. A small residual antigen presence can still be detected in Peyer’s patch tissues at that time (2). Persistent antigen may be responsible for the continual induction of memory B cells to form plasma cells.

The specificities of primary and memory responses have, in some cases, been shown to differ (1, 10, 11). A set of idiotypically related hemagglutination-specific B cells that are prominent in the primary BALB/c murine response to influenza virus are absent from the secondary response (3). We did not perform such precise measures of the responding antibody idiotypic repertoire, but we did compare the boosted antibody response to a variety of rotavirus antigens (intact double-encapsidated rotavirus, single-capsid virus lacking VP4 and VP7, and a baculovirus-expressed VP4) with the relative responses we observed in the primary response (data not shown) (5). The response at 360 days without boosting and the response after boosting were both dominated by inner capsid-specific antibodies (presumably VP6 specific) and directed less than 1% of virus-specific antibodies toward VP4. Thus, we were unable to detect.
evidence of a shift in protein specificity of the intestinal antibody response over time.

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REFERENCES