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Circulating Immunoglobulin G Can Play a Critical Role in Clearance of Intestinal Reovirus Infection

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Reoviruses are encapsidated double-stranded RNA viruses that cause systemic disease in mice after peroral (p.o.) inoculation and primary replication in the intestine. In this study, we define components of the immune system involved in the clearing of reovirus from the proximal small intestine. The intestines of immunocompetent adult CB17, 129, and C57BL/6 mice were cleared of reovirus serotype 3 clone 9 (T3C9) within 7 days of p.o. inoculation. Antigen-specific lymphocytes were important for the clearance of intestinal infection, since severe combined immunodeficient (SCID) mice failed to clear T3C9 infection. To define specific immune components required for intestinal clearance, reovirus infection of mice with null mutations in the immunoglobulin M (IgM) transmembrane exon (MuMT; B cell and antibody deficient) or β2 microglobulin gene (β2-/-; CD8 deficient) was evaluated. β2-/- mice cleared reovirus infection with normal kinetics, while MuMT mice showed delayed clearance of T3C9 7 to 11 days after p.o. inoculation. Adoptive transfer of splenic lymphocytes from reovirus-immune CB17 mice inhibited growth of T3C9 in CB17 SCID mouse intestine 11 days after p.o. inoculation. The efficiency of viral clearance by adoptively transferred cells was significantly diminished by depletion of B cells prior to adoptive transfer. Results in SCID and MuMT mice demonstrate an important role for B cells or IgG in clearance of reovirus from the intestines. Polyclonal reovirus-immune rabbit serum, protein A-purified immune IgG, and murine monoclonal IgG2a antibody specific for reovirus outer capsid protein σ3 administered intraperitoneally all normalized clearance of reovirus from intestinal tissue in MuMT mice. This result demonstrates an IgA-independent role for IgG in the clearance of intestinal virus infection. Polyclonal reovirus-immune serum also significantly decreased reovirus titers in the intestines of SCID mice, demonstrating a T-cell-independent role for antibody in the clearance of intestinal reovirus infection. B cells and circulating IgG play an important role in the clearance of reovirus from intestines, suggesting that IgG may play a more prominent functional role at mucosal sites of primary viral replication than was previously supposed.

Reoviruses are encapsidated double-stranded RNA viruses whose pathogenesis has been extensively analyzed (42, 53). Two model systems, infection of neonatal outbred mice and infection of adult severe combined immunodeficient (SCID) mice, have been characterized (19, 20, 51, 53). In the neonatal model, reoviruses inoculated perorally (p.o.) are taken up via M cells overlying Peyer's patches and replicate in mononuclear cells (reviewed in references 41 and 53). Subsequently, virus spreads to adjacent intestinal tissue by infecting the basal surfaces of intestinal epithelial cells (47, 48, 60) and entering myenteric neurons (37, 38). Such viruses as serotype 3 clone 9 (T3C9) then spread to the brain via the vagus nerve (38) and ultimately cause a lethal meningoencephalitis (53). Similarly, reovirus serotype 3 strain Dearing (T3D) enters the inferior spinal cord via the sciatic nerve after intramuscular inoculation (55) to cause lethal meningoencephalitis. The effect of antibody at these defined stages of pathogenesis has been evaluated in some detail. Antibody (immunoglobulin G [IgG]) protects neonatal NIH Swiss strain mice against lethal infection with neurotropic reoviruses T3D and T3C9 and prevents hydrocephalus and myocarditis induced by other reoviruses (51, 54, 56, 57). Monoclonal antibody (MAb)-mediated protection against T3D after intramuscular inoculation in the hind limb is associated with the inhibition of spread to the inferior spinal cord, in some cases without effects on primary replication (54, 56, 57). IgG MAbs inhibit the spread of T3C9 via the vagus nerve to the brain, in many cases without altering T3C9 titers in the intestine (54, 56). These studies show that IgG can block neural spread from two different primary inoculation sites early after infection, when effects on primary replication are minimal or absent.

While it is known that IgG can protect against systemic disease in these models, little is known about the mechanisms responsible for the control of viral replication and final clearance of virus from the primary site of infection in the intestine. In several other viral systems, IgG has been shown to have a role in the clearance of or prevention of infection at mucosal surfaces (3, 6, 40, 44). While adoptive transfer of protective MAbs protects neonatal NIH Swiss mice against lethal p.o. challenge with T3C9, this protection was not associated with the control of viral replication in the intestine 5 days after p.o. inoculation (54, 56). This result argues that the protective effects of IgG in neonatal mice are not due to the effects on infection in the intestine. In other studies, both secretory and transplacental factors from immune dams protected neonates against lethal p.o. reovirus infection, and in some cases, this protection was accompanied by a decreased detection of virus

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in the intestine (10). This result demonstrates that immunity, and likely antibody, can prevent and/or control reovirus infection in the intestine. Other studies have shown a vigorous cellular response to reovirus in intestinal tissues. In the intraepithelial lymphocytes and Peyer's patches of adult mice, reovirus infection elicits a CD8 cytotoxic T-cell response in addition to a significant IgA plasma cell response (32–34). In addition, adoptive transfer of immune spleen cells to neonatal mice is more effective than single doses of MAb in controlling T3C9 replication in neonatal intestines (59), and adoptive transfer of intestine-derived lymphocytes protects against lethal infection (9). However, depletion of CD4 and/or CD8 T cells from neonatal mice had little effect on reovirus titers in the intestine (59). Thus, studies to date have not definitively addressed the roles of cellular and humoral responses in the clearance of reovirus infection from the intestine.

In this report, we address the importance of lymphocytes, antibody, B cells, and CD8 T cells in the clearance of reovirus from adult intestines. CD8 T-cell deficiency (secondary to a null mutation in β2 microglobulin) had no effect on intestinal clearance, while the absence of antigen-specific lymphocytes in SCID mice abrogated clearance of intestinal virus infection. The absence of an effective antibody response in B-cell-deficient MuMT mice led to the delayed clearance of virus from the intestine. Polyclonal reovirus-immune spleen cells enhanced reovirus clearance from SCID mice, and depletion of B cells from these immune spleen cells removed this protective effect. Adoptive transfer of antireovirus IgG (rabbit or mouse) enhanced intestinal clearance of reovirus in SCID mice and normalized reovirus clearance in MuMT mice. We argue from these observations that IgG can play an important role in viral clearance from the primary site of infection in the intestine, and we speculate that IgG may act in the intestine by inhibiting infection in the lamina propria.

MATERIALS AND METHODS

Viruses and tissue culture. All tissue culture media contained 100 U of penicillin per ml and 100 μg of streptomycin per ml (Biofluids, Rockville, Md., or Irvine Scientific, Santa Ana, Calif.). Mouse L929 fibroblasts were maintained in spinner culture in minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS) (Hyclone, Ogden, Utah, or Biofluids) and 2 mM L-glutamine (Biofluids) (MEM+). T3C9 and T3D were from laboratory stocks that were originally a gift from B. Fields. Viruses were plaque purified twice, passaged twice in L cells, and stored in MEM+ at 4°C before inoculation into mice. Methods for virus growth, storage, and plaque assays were as described previously (54, 56, 57). Titers of the virus were determined by plating serial 10-fold dilutions of virus samples on L929 fibroblast monolayers, overlaying them with agar, and staining them with neutral red. T3D was purified on cesium chloride gradients for immunization and enzyme-linked immunosorbent assay (ELISA) (57, 58).

Mice and inoculations of mice. Mice were housed and bred at the Washington University School of Medicine at biosafety level 2 in accordance with all federal and university policies or were purchased from the Jackson Laboratory (Bar Harbor, Maine). Sentinel mice screened negative every 2 to 4 months for adventitious mouse pathogens. β2-/- and MuMT mice were obtained from D. Roopenian and L. Schultz, respectively, at the Jackson Laboratory. β2-/- mice were derived by disruption of the β2 microglobulin gene and have a significant deficiency in the number and function of CD8 T cells (28). We confirmed CD8 T-cell deficiency in our β2-/- mice by fluorescence-activated cell sorter (FACS) analysis (data not shown). MuMT mice were derived by interruption of a transmembrane exon of IgM and are deficient in functional B cells (27). We confirmed the expected B-cell deficiency in MuMT mice by FACS analysis (data not shown). β2-/- mice made a significant anti-reovirus IgG response to infection, while MuMT mice did not (data not shown). Breeder pairs of C57BL/6 SCID mice were purchased from the Jackson Laboratory. Since both MuMT and β2-/- mice carry a combination of 129 and C57BL/6 genes, 129/J or 129Ev/Sv and C57BL/6 mice were used as immunocompetent controls. CB17 and CB17 SCID mice were bred at Washington University.

Mice were inoculated p.o., with soft polyethylene tubing being used to place 4.5×10^7 PFU of reovirus T3C9 in 0.5 ml of MEM+ intragastrically (56). Intraperitoneal (i.p.) challenge was with 4.5×10^7 PFU of T3C9 in 1.0 ml of MEM+. Adult mice from 1 to 11 months old were used for all experiments. At different times after infection, a piece of liver (1 by 1 cm) and approximately 2 cm of intestine distal to the gastric antrum were harvested in 1.0 ml of gel saline (20, 57). Tissue samples were frozen and thawed three times prior to sonication and titration by plaque assay. Unless otherwise noted, data represent mean log titers. Since the standard errors of the means for different experimental groups (see Fig. 1 and 2) were never greater than 20% of the means, these data are presented without error bars. The number of mice and number of experimental repeats are reported in the figure legends.

Antibodies and adoptive transfer protocols. MAbs 4A3 (IgG2b, specific for the μ1 reovirus outer capsid protein) and 10C1 (IgG2a, specific for the reovirus σ3 outer capsid protein) were protein A purified as previously described (58). Preimmune and postimmune anti-reovirus rabbit serum was prepared by immunizing rabbits with cesium chloride-purified T3D in complete Freund's adjuvant (57). Purified polyclonal rabbit IgG specific for T3D was prepared by protein A chromatography (57). The adoptive transfer of antibodies was performed i.p. 3 and 5 days after p.o. challenge with T3C9. The doses of antibody were (i) 0.25 ml of serum, (ii) 0.25 mg of purified polyclonal rabbit IgG specific for T3D, or (iii) 0.25 mg of either 4A3 or 10C1. Eleven days after the initial inoculation, the mice were sacrificed and samples of liver and intestine were collected for titration of virus.

Adoptive transfer of splenic lymphocytes and depletion of B cells by panning. Plates for panning were coated with antibody in the following way. Five milliliters of 5 μg of goat anti-mouse IgG plus IgM per ml (TAGO, Burlingame, Calif.) (to deplete B cells) or a mixture of rat immunoglobulin plus goat gamma globulin (control) diluted in 0.05 M Tris-HCl–0.15 M NaCl (pH 9.5) was added to bacteriologic petri dishes (100 by 15 mm; Falcon, Lincoln Park, N.J.) and stored at 4°C overnight. Dishes were washed three times with phosphate-buffered saline (PBS) and blocked with 10 ml of PBS containing 5% FCS per dish for 10 to 15 min at room temperature. Spleen cells were collected from CB17 mice immunized with either T3D in complete Freund's adjuvant (CFA) or CFA alone and prepared as described previously (51, 59). Previous work showed that T3D and T3C9 are extensively cross-reactive serologically and that T3D-immune cells protect against T3C9 (54, 58, 59). Briefly, spleens were disrupted, filtered to remove splenic stroma, treated with hypotonic buffer to lyse erythrocytes, and adjusted to 5×10^6 cells per ml in PBS containing 5% FCS. After the blocking buffer was removed from the dishes, 5 ml of spleen cells per dish containing a total of 2.5×10^7 cells was added and incubated at room temperature for 70 min with occasional swirling. Nonadherent cells were collected by pipetting 10 ml of PBS containing 5% FCS five times over the surface of the plates, washing the plates once, and subjecting them to a second round of panning. Nonadherent cells from the second round of panning were resuspended in RPMI 1640 medium containing 10% FCS and adoptively transferred to SCID mice by i.p. injection (5×10^6 cells per mouse in 1.0 ml). Twenty-four hours later, the mice were infected p.o. with 4.5×10^7 PFU of T3C9 per mouse. To characterize the efficacy of panning, the cells were analyzed by FACS (Becton Dickinson, Lincoln Park, N.J.) as previously described (21). The antibodies used included rat immunoglobulin (negative control) and rat MAbs GK1.5 (specific for CD4 [13]) and 53.6-72 (specific for CD8 [29]), and they were prepared as described previously (59). Rat MAbs were detected with fluorescein-coupled goat anti-rat IgG heavy and light chains (Caltag, San Francisco, Calif.). B cells were detected with fluorescein-conjugated goat anti-mouse IgG plus IgM.

ELISA. Anti-reovirus antibody was detected by ELISA as described previously, with the exception that 100 μg of purified T3D per ml was used to coat the wells (58, 59). Serum was collected from mice at the time of sacrifice by cardiac puncture. Serial 0.5-log dilutions starting at 1:10 were made in 150 mM NaCl–1 mM EDTA–50 mM Tris–0.05% Tween 20 (pH 7.4) and then were added to wells in duplicate. Bound antibody was detected with a 1:200 dilution of protein A-peroxidase (Zymed, San Francisco, Calif.) and colorimetric substrate 2,2′-azino-bis(3-ethylbenz thiazoline-6-sulfonic acid) (Sigma, St. Louis, Mo.). The data are presented as the mean optical density at 405 nm at a given dilution. The numbers of experiments and experimental animals analyzed are presented elsewhere (see the legend to Fig. 3).

Statistical analysis. All analyses were performed with $\log_{10}$s of viral titer (20). For purposes of comparison, titer values below the plaque assay level of sensitivity (10^2 PFU/ml) were set equal to 10^2 ($\log_{10}$ titer = 2.0) for comparison with other values. For experiments using adoptive transfer of cell populations to SCID mice (see Fig. 3), titers within a group ranged from below to above plaque assay sensitivity. In this case, we applied a Wilcoxon rank sum test, assigning all values below assay detection to 2.0. For experiments with MuMT mice (see Fig. 4), statistical analysis could not be performed, since all values in mice receiving anti-reovirus antibody were below assay sensitivity. The lack of detectable virus in each of the animals receiving immune antibody invalidates assumptions about the normal distribution of titer data required for valid statistical analysis. These data are therefore presented without statistical analysis. For experiments with SCID mice (see Fig. 4), groups were compared by analysis of variance (20).

RESULTS

Clearance of virus in normal and immunocompromised mice. To define components of the immune system important for clearance of reovirus from the intestine, we infected normal

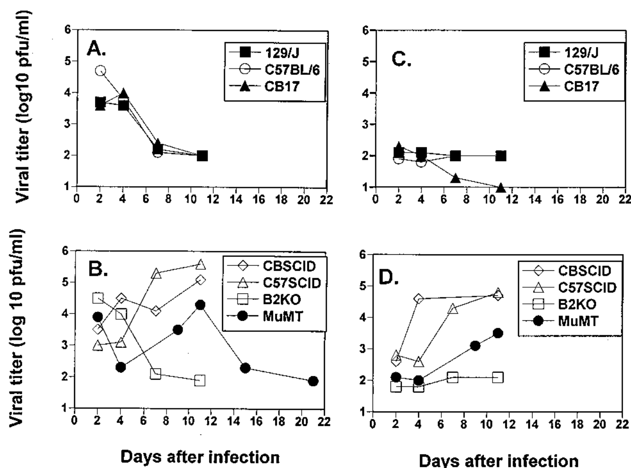

FIG. 1. Reovirus titers in the livers and intestines of mice from various strains after p.o. inoculation. Different groups of mice, as indicated in the inset legends, were inoculated p.o. with 4.5×10^7 PFU of reovirus T3C9. Organs were harvested from two to four mice per time point, and viral titers were determined. (A and B) Viral titers in the intestines of immunocompetent and immunocompromised mice, respectively. (C and D) Viral titers in liver tissues of immunocompetent and immunocompromised mice, respectively.

and immunocompromised mice either i.p. or p.o. with 4.5×10^7 PFU of T3C9 and evaluated viral titers in the liver and intestine at various times after infection (Fig. 1 and 2). We evaluated both i.p. and p.o. infections in order to assess the effect of the route of inoculation and to evaluate clearance from the liver since no systemic spread of reovirus infection occurred in normal mice after p.o. infection (Fig. 1). The resistance of normal adult mice to systemic reovirus infection (Fig. 1) has been previously reported (52). After i.p. challenge, hepatic infection was established even in normal mice, but virus was rapidly cleared (Fig. 2). All immunocompetent mice (129/J, C57BL/6, and CB17) cleared T3C9 from both the intestine and liver by 7 to 11 days after either p.o. or i.p. challenge (Fig. 1A and C and 2A and C). In contrast, SCID mice on either the CB17 or C57BL/6 background failed to effectively clear virus after either p.o. or i.p. infection (Fig. 1B and D and 2B and D). Eleven days after infection, reovirus titers in SCID mouse intestine and liver were 600- to 1,200-fold higher and 500- to 2,500-fold higher, respectively, than those in control mice. The failure of SCID mice to control reovirus infection is consistent with previous reports (19, 20, 51) and demonstrates that an effective antigen-specific lymphocyte response is important for clearance of reovirus infection from the intestine.

Since both CD8 and IgA responses to reovirus have been documented in intestinal tissues after p.o. challenge with reovirus, we next compared intestinal reovirus clearances in β2-/- mice (deficient in β2 microglobulin and CD8 T cells [28]) and MuMT mice (deficient in mature B cells and immunoglobulin [27]). β2-/- mice cleared virus from the intestine at the same rate as immunocompetent mice did (Fig. 1B and D and 2B and D), demonstrating that β2 microglobulin is not essential and suggesting that CD8 T-cell responses are not essential for intestinal clearance between 7 and 11 days after infection. In contrast to β2-/- mice, MuMT mice did not clear the virus until days 15 to 21, and increases in viral titers were seen between 7 and 11 days after p.o. challenge (Fig. 1B and D and 2B and D). On day 11, reovirus titers in the livers of MuMT mice were 32- to 212-fold higher than those in control mice, while intestinal titers were 200-fold higher than those in controls. The titers in MuMT mice did not reach the levels seen in SCID mice (Fig. 1 and 2), suggesting that some factor in addition to B cells contributes to the control of intestinal virus titers. After day 11, reovirus titers in the intestines of MuMT mice decreased, although we intermittently detected reovirus in the intestines of MuMT mice as long as 21 days after infection (Fig. 1B). Since clearance patterns are similar after i.p. and p.o. inoculation, the immune competence of the mouse rather than the route of infection is the primary determinant of the efficiency of viral clearance from the intestine. These data show that either B cells or antibody is important for the clearance of reovirus infection from the intestine 11 days after infection. Since MuMT mice can control reovirus titers in the intestine to some extent (compare MuMT titers at day 11 and those at day 22 in Fig. 1B), B-cell- and antibody-independent mechanisms must also be involved in clearance at later times.

Clearance of reovirus from intestines by T3D-immune spleen cells. To determine the importance of B cells and antibody, we devised an independent experimental system utilizing the adoptive transfer of immune or control spleen cells to SCID mice. Splenic lymphocytes from CB17 mice immunized with purified reovirus T3D in CFA were adoptively transferred to CB17 SCID mice 24 h before p.o. challenge with T3C9, and viral titers were evaluated 11 days later (Fig. 3A). Adoptive transfer of 5×10^6 immune cells decreased reovirus titers in the intestine 250- to 790-fold compared with no adoptive transfer and adoptive transfer of cells from CFA-immunized mice (Fig. 3A).

To determine if B cells are important for the clearance of virus from the intestine in this model, spleen cells depleted of B cells by panning to remove surface immunoglobulin-bearing cells were adoptively transferred to SCID mice (Fig. 3A). Panning with antibody specific for mouse immunoglobulin depleted an average of 98% of B cells across three experiments, with the expected compensatory increase in the proportion of CD4 and CD8 T cells (Fig. 3B). Panning with control antibodies did not remove B cells (Fig. 3B). We assessed the functional efficacy of B-cell depletion by measuring the anti-reovirus antibody response in SCID recipients of various spleen cell populations (Fig. 3C). Adoptive transfer of 5×10^6 immune cells and 5×10^6 control-panned immune cells resulted in detectable anti-reovirus antibody in SCID mice 11 days after p.o.

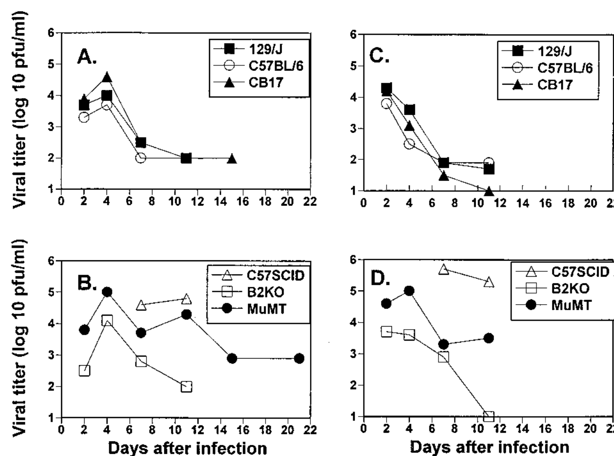

FIG. 2. Reovirus titers in the livers and intestines of mice from various strains after i.p. inoculation. The experiment was performed and the data are presented as described in the legend to Fig. 1, except that the virus was administered i.p. (A and B) Viral titers in the intestines of immunocompetent and immunocompromised mice, respectively. (C and D) Viral titers in liver tissues of immunocompetent and immunocompromised mice, respectively.

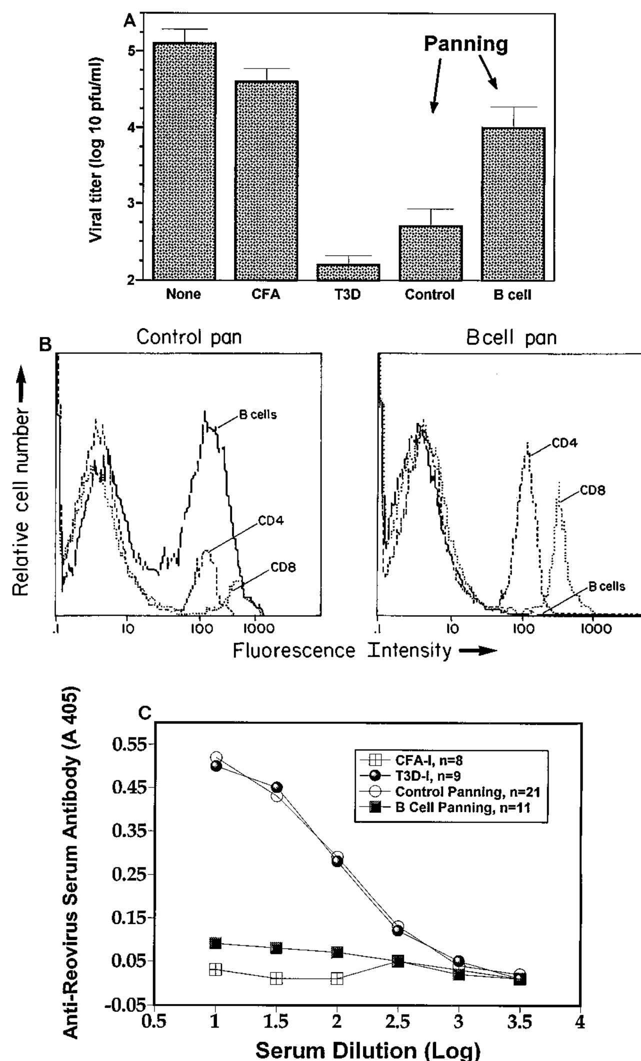

FIG. 3. Effect of adoptive transfer of reovirus-immune splenic lymphocytes on viral titers in SCID mouse intestines. (A) SCID mice were adoptively transferred with either no cells (None) or 5×10^6 spleen cells per mouse 24 h prior to p.o. inoculation with 4.5×10^7 PFU of reovirus T3C9. Eleven days later, the intestines were harvested and the titers of virus in intestinal tissues were determined. The data show the means ± the standard errors of the means for 8 to 21 SCID mice pooled from three to five different experiments. Lymphocytes were isolated from immunocompetent CB17 mice immunized with either T3D in CFA (T3D) or CFA alone (CFA). T3D-immune cells were panned with control or B-cell-depleting antibody and then were adoptively transferred. (B) FACS profiles for control-panned and B-cell-panned lymphocytes transferred to SCID mice from one of the three pooled experiments are shown (Fig. 3A). The cell type corresponding to each curve is noted in each panel. (C) ELISA data indicating the amount of anti-reovirus IgG present in serum of SCID mice receiving various cells by adoptive transfer 11 days after p.o. infection with T3C9 are shown. The data represent the mean optical density at 405 nm at each 0.5-$\log_{10}$ dilution for the number of mice in each experimental group shown in the inset. The groups correspond to the groups shown in Fig. 3A.

challenge with T3C9. In contrast, depletion of B cells decreased the anti-reovirus response to levels similar to those seen after adoptive transfer of CFA-immune cells (Fig. 3C).

Control-panned cells effectively decreased reovirus titers in the intestine 11 days after p.o. inoculation ($P < 0.0001$ for the difference between CFA-immune and control-panned cells) (Fig. 3A). In contrast, immune spleen cells depleted of B cells were about 20-fold less effective than control-panned cells at decreasing reovirus titers in intestines 11 days after p.o. infection with T3C9 ($P = 0.0013$ for the difference between control-panned and B-cell-panned cells) (Fig. 3A). The difference between B-cell-panned and CFA-immune cells (Fig. 3) was not statistically significant ($P = 0.085$). These data confirm results with MuMT mice by showing that B cells were important for the clearance of reovirus infection from the intestine.

Virus clearance in intestines of MuMT and SCID mice after passive transfer of antibody. Since two different approaches defined B cells as important for clearance of reovirus from intestines, we determined whether antibody alone could substitute for B cells. Antibody was passively transferred i.p. 3 and 5 days after p.o. inoculation of SCID and MuMT mice with T3C9. We delayed antibody transfer until after the establishment of intestinal infection so that data would reflect control of established infection rather than prevention of infection. Intestines were collected for viral titers 11 days after infection (Fig. 4). MuMT mice given preimmune serum had significant titers of reovirus in the intestines 11 days after infection, whereas mice receiving immune serum had no detectable virus (Fig. 4). This outcome represents an at least 60-fold decrease in viral titers compared with titers for untreated controls. This decrease is likely an underestimate, since viral titers after the transfer of immune serum were below our level of detection.

Since rabbit serum contains both IgG and IgA, we determined whether IgG could confer enhanced clearance of virus from intestines by passively transferring protein A-purified polyclonal immune IgG and murine IgG MAbs specific for reovirus outer capsid proteins. MuMT mice receiving either polyclonal reovirus-immune IgG or MAb 10C1 had no detectable virus in the intestines 11 days after infection. 10C1 is an IgG2a MAb specific for the σ3 outer capsid protein and is protective against reovirus-induced lethal infection or hydrocephalus in neonatal mice (54). In contrast, mice receiving control IgG (MAb 4A3, which is not protective in neonatal mice [54]) had significant reovirus titers in the intestines (Fig. 4). Thus, passive transfer of systemic IgG normalized reovirus clearance in MuMT mice. When passive transfer experiments were performed with SCID mice, polyclonal immune serum limited intestinal reovirus titers but did not decrease titers as

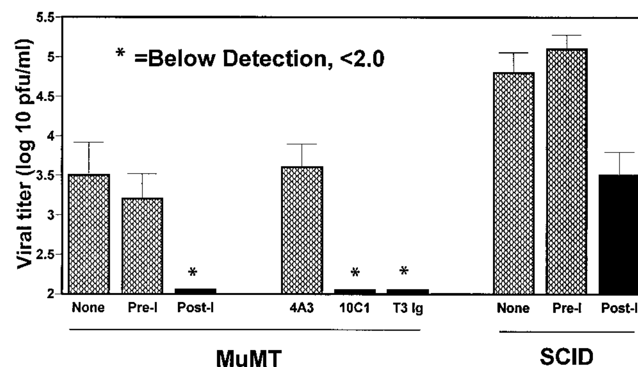

FIG. 4. Effect of passive transfer of antibody on reovirus titers in the intestines of SCID and MuMT mice. Mice received various antibody preparations as described in Materials and Methods and were then challenged with 4.5×10^7 PFU of T3C9 p.o. Eleven days after infection, intestines were harvested and the titers of virus in intestinal tissues were determined. Viral titers shown represent the mean $\log_{10}$ titers ± the standard errors of the means for six to eight mice pooled from two independent experiments. MuMT mice are deficient in B cells, while SCID mice have no functional T or B cells. Pre-I, preimmune polyclonal rabbit serum; Post-I, polyclonal rabbit anti-T3D serum. 4A3 is a murine IgG MAb specific for the reovirus μ1 outer capsid protein, which is not protective in neonatal mice. 10C1 is a murine IgG MAb specific for the reovirus σ3 outer capsid protein, which is protective in neonatal mice. T3 Ig refers to IgG purified from rabbit polyclonal anti-T3D serum with protein A.

efficiently as did the same transfer to MuMT mice (compare pre- and postimmune serum levels in MuMT and SCID mice [Fig. 4]). The difference in intestinal titers for SCID recipients of preimmune serum and postimmune serum was significant at the $P = 0.0005$ level. The difference in antibody efficiency for SCID and MuMT mice argues that an immune system component (e.g., T cells) is operative in MuMT mice but absent in SCID mice, with the result that passively transferred antibody is less effective in SCID mice. Together, these experiments show that polyclonal serum as well as affinity-purified rabbit IgG and monoclonal murine IgG antibodies administered i.p. significantly enhanced clearance of reovirus from intestines.

DISCUSSION

In this paper, we demonstrate that IgG can play an IgA-independent role in the clearance of virus from mucosal (intestinal) primary sites of infection. IgA has been considered a primary mediator of resistance to infection at this mucosal site, and thus much vaccine research has focused on the generation of mucosal IgA responses to viruses which enter via the gastrointestinal tract. Our finding that IgG can play an important role at this mucosal site suggests that it is worth reconsidering paradigms of mucosal immunity. In particular, it may be reasonable to consider generation of optimal systemic IgG responses as an important goal for control of virus infections at mucosal sites.

Antibody action in clearance of primary reovirus infection of the intestine. The first step in pathogenesis at the level of the mucosa is gaining entry into a site at which primary replication can occur. For reoviruses, this step involves proteolysis of the outer capsid of the virion to generate infectious subviral particles which can then bind M cells and enter the host (1, 41, 62). In the luminal milieu, multiple substances might be involved in preventing this critical step. It is a reasonable hypothesis that secretory IgA plays a prominent role in this regard, although no direct data addressing this hypothesis in the reovirus system exist. Under experimental conditions, anti-reovirus IgA MAbs as well as IgG antibodies bind to M cell surfaces and are transported into the cell-containing pocket of the M cell (61). Thus, both IgG and IgA may be present at critical sites in the normal intestine during reovirus infection. The direct relevance of these studies to results presented here remains to be examined, since both MuMT and SCID mice lack Peyer's patches (unpublished observation) (11) and since the route of reovirus entry into the intestine in these immunocompromised mice is not known.

The second step in pathogenesis is establishing infection at a site of primary replication, in this case, in intestinal tissue. This step is critical for two reasons. First, it provides sufficient virus for systemic invasion and subsequent generation of systemic disease. Second, reovirus replication in the intestine is the most likely source of virus shed into the intestine and thus is likely related to the horizontal spread of virus to new hosts (4, 26). For reovirus, this step in pathogenesis involves the infection of a number of cell types present in the intestine, including M cells, mononuclear cells, absorptive cells, and myenteric neurons (37, 38, 41, 47, 48, 53, 60). Immune control of this stage in intestinal pathogenesis is critical both to protection of the host and to decreasing the horizontal spread of the virus. It is this step which we have analyzed in the studies presented here.

Multiple types of responses are elicited in intestinal tissue during reovirus infection. In particular, both prominent IgA plasma cell and CD8 T-cell responses occur (5, 32–34). We used mice with null mutations to determine the importance of these two types of responses (B cells and CD8 T cells) in the clearance of intestinal infection. Replication of reovirus in MuMT mice 7 to 11 days after p.o. infection (Fig. 1 and 2), combined with the importance of B cells in the control of intestinal reovirus replication after adoptive transfer to SCID mice (Fig. 3), shows that B cells are important for the control of replication in the intestine. The normal clearance of reovirus in CD8-deficient mice is discussed in more detail below. The fact that clearance of reovirus intestinal infection in MuMT mice could be completely restored by passive transfer of IgG antibody (affinity purified or monoclonal) demonstrates that circulating IgG can play an IgA-independent role in the clearance of intestinal infection. While these data show that IgG can contribute to the clearance of reovirus infection from B-cell-deficient mice, our experiments do not address whether IgA could also play such a role. These findings contrast with those of previous studies in which passive transfer of protective MAbs to neonatal outbred mice had no effect on reovirus replication in intestines 5 days after infection (55, 56). This apparent difference could be due to differences between experimental systems (neonatal outbred versus adult inbred mice) and to the fact that viral clearance at time points after 5 days was not assessed in these previous studies.

It is possible that antibody plays an indirect role in promoting the clearance of virus in MuMT mice, because it is required for mounting an effective cellular (e.g., CD4 or CD8) response to reovirus infection. However, the fact that circulating antibody can contribute to control of reovirus replication in the intestinal tissue of SCID mice argues that antibody has direct actions in the intestine independent of T cells and B cells.

We have not defined the site(s) at which IgG can act to enhance the clearance of reovirus from the intestine. Since reovirus infects intestinal epithelial cells via the basolateral surfaces (47, 48, 60), one hypothesis to explain our findings is that IgG acts in the lamina propria. In this way, IgG could enhance viral clearance in the intestine and even decrease reovirus shedding into the lumen by limiting the infection of epithelial (or other) cells critical for virus replication. Studies are currently under way to determine the localization of virus-infected cells in the intestines of MuMT mice in the presence and absence of passively transferred IgG in order to address this question.

Role for CD4 and CD8 T cells in the intestine. Both CD4 and CD8 T cells have been shown to have a role in protection against visceral disease (either encephalitis or myocarditis) due to reoviruses (51, 59). This fact, combined with the demonstration that oral infection with reovirus elicits a cellular immune response in intestinal tissue (5, 32–34), argues for an important role for these responses in the intestine. In an adoptive transfer system similar to that reported here, purified immune CD8 T cells played an important role in the clearance of rotavirus infection from SCID mouse intestines in the absence of detectable antibody responses (12). However, in contrast to the role of B cells and antibody in controlling intestinal reovirus infection, mice deficient in CD8 T cells (β2-/-) were capable of clearing intestinal reovirus infection with normal kinetics. This result was surprising to us and demonstrates that expression of β2 microglobulin is not essential for reovirus clearance from the intestine.

Normal reovirus clearance in β2-/- mice suggests but does not prove that CD8 T cells are not essential for reovirus clearance from the intestine. However, interpretation of this negative result comes with three caveats. First, titers with MuMT mice were lower than those with SCID mice (Fig. 1 and 2), which indicates that a component of the immune system (perhaps CD8 T cells) in addition to B cells and antibody was active in the intestine. Second, it is possible that the lack of CD8 T

cells in β2-/- mice has led to a compensatory increase in some other aspect of intestinal immunity (such as local antibody secretion), with the result that a role for CD8 T cells in the control of intestinal virus infection is obscured in β2-/- mice. In fact, β2-/- mice develop a larger increase in IgA-secreting B cells than normal mice in response to Sendai virus infection (25), and CD8-deficient mice produce more neutralizing antibody than control animals when infected with some but not all viruses (2, 31, 46). Since we have demonstrated a critical role for antibody responses in intestinal clearance in this study, an increase in intestinal antibody secretion in β2-/- mice could obscure an important role for CD8 T cells. It has also been argued that a compensatory CD4 cytotoxic T-lymphocyte response is seen in β2-/- mice in response to lymphocytic choriomeningitis virus or Sendai virus (22, 39), although a possible role for CD4 cytotoxic T lymphocytes has not been evaluated in the reovirus system. Lastly, the significant deficiency of CD8 T cells in β2-/- mice (14, 22, 28, 31, 36, 39) may not be absolute, since major histocompatibility complex class I-restricted CD8 T cells have been cloned from β2-/- mice and adoptive transfer studies show that viral antigen can be presented in vivo to CD8 T cells in β2-/- mice (7, 30). Thus, it is possible that residual CD8 T cells play some role in reovirus clearance. It should be noted, however, that the extent of CD8 deficiency in β2-/- mice is sufficient to alter the clearance of many viruses (e.g., lymphocytic choriomeningitis virus, Sendai virus, and Theiler's virus) for which CD8 T cells play an important role (14, 16, 23, 31, 36, 46).

Our data suggest that some aspect of immunity in addition to that of B cells and antibody plays a role in the intestinal clearance of reovirus. Thus, while MuMT mice could not control reovirus replication 7 to 11 days after infection, they could control reoviral replication after approximately 15 to 20 days. We expect that this delayed clearance is likely to be T cell mediated. Evaluation of the mechanisms of viral clearance in the absence of functional B cells may reveal roles for CD8 and/or CD4 T cells. Evaluation of mice carrying null alleles interrupting multiple arms of the immune system may prove informative in evaluating whether CD4 or CD8 T cells, in addition to B cells and antibody, play a role in reovirus clearance.

Paradigms of mucosal immunity in the intestine. Reovirus infection serves as a model for the analysis of the pathogenesis of viruses which enter via the gastrointestinal tract, replicate in cells of the intestine, and then spread to visceral organs and the central nervous system to cause disease. The relevance of IgG responses in the intestine in the control of infection with an agent using this type of strategy has not received extensive attention. This neglect is largely due to studies with the poliovirus system demonstrating an association between mucosal IgA induction and the efficacy of live attenuated poliovirus vaccines in controlling intestinal virus shedding. However, while the majority of antibody secreted by intestinal B cells is IgA, a large amount of IgG is also produced (45).

For poliovirus, efficient control of intestinal shedding is seen after p.o. inoculation with live attenuated virus (Sabin). It has been argued that the control of primary replication at mucosal sites is less effective after systemic inoculation with killed virus (Salk) (reviewed in reference 17). Since live attenuated virus elicits a secretory IgA response, it has been assumed that secretory IgA is an important determinant of this difference in the efficacies of mucosal immunity for the Salk and Sabin vaccines (43). However, several studies with the poliovirus system suggest that Salk vaccination does result in efficient control of viral infection at mucosal sites, despite its inefficiency in inducing mucosal IgA. First, the incidence of poliovirus infection decreased after the introduction of the Salk vaccine and before widespread use of the Sabin vaccine, suggesting a role for systemic immunization in controlling spread within a population (49, 50). For example, in a family cohort in Alabama, introduction of the Salk vaccine was associated with an unexpected decrease in new cases of poliovirus infection (18). In chimpanzees, vaccination with killed virus can decrease oropharyngeal and fecal shedding of the virus (24). In another study, inactivated vaccine decreased pharyngeal shedding of poliovirus, but oral immunization with live homologous virus was more effective (8). A decrease in both fecal and pharyngeal shedding of poliovirus was also seen with Salk-vaccinated persons in a study conducted during a type 1 poliovirus epidemic (35). These studies, taken together, have consistently raised the possibility that factors in addition to IgA are important for the intestinal clearance of virus infection.

The data presented here show that systemic IgG can play an important role in the clearance of intestinal virus infection and thus provide a mechanism to explain the effects of Salk vaccination on poliovirus spread and mucosal shedding. The fact that live attenuated vaccination is more effective than killed parenteral vaccination at controlling mucosal shedding could be due to an additive effect of IgA plus IgG or, alternatively, more effective generation of mucosal cellular immunity by live vaccination. An important role for IgG in the lower respiratory tract has also been shown in some studies (reviewed in reference 40). In the rotavirus system, intestinal IgA responses correlate with protection after oral immunization (15). However, immunization studies and passive transfer studies have indicated that IgG can be an important determinant of intestinal rotavirus infection (3, 6). These studies, and the demonstration here that systemic IgG can play an IgA-independent role at mucosal surfaces, argue for direct experimental evaluation of the mechanisms by which IgA, IgG, and cellular responses result in the clearance of virus from the intestinal portal of entry.

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