

## Role of Immunoglobulin A in Protection against Reovirus Entry into Murine Peyer's Patches†

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**Reovirus type 1 Lang (T1L) infects the mouse intestinal mucosa by adhering specifically to epithelial M cells and exploiting M-cell transport to enter the Peyer's patches. Oral inoculation of adult mice has been shown to elicit cellular and humoral immune responses that clear the infection within 10 days. This study was designed to determine whether adult mice that have cleared a primary infection are protected against viral entry upon oral rechallenge and, if so, whether antireovirus secretory immunoglobulin A (S-IgA) is a necessary component of protection. Adult BALB/c mice that were orally inoculated on day 0 with reovirus T1L produced antiviral S-IgA in feces and IgG in serum directed primarily against the reovirus  $\sigma 1$  attachment protein. Eight hours after oral reovirus challenge on day 21, the Peyer's patches of previously exposed mice contained no detectable virus whereas Peyer's patches of naive controls contained up to 2,300 PFU of reovirus/mg of tissue. Orally inoculated IgA knockout (IgA<sup>-/-</sup>) mice cleared the initial infection as effectively as wild-type mice and produced higher levels of reovirus-specific serum IgG and secretory IgM than C57BL/6 wild-type mice. When IgA<sup>-/-</sup> mice were rechallenged on day 21, however, their Peyer's patches became infected. These results indicate that intestinal S-IgA is an essential component of immune protection against reovirus entry into Peyer's patch mucosa.**

Secretory immunoglobulin A (S-IgA) is the predominant immunoglobulin on the intestinal mucosal surface and is considered to be a first line of immune defense, protecting the mucosa against adherence and invasion by enteric pathogens (32). There is evidence that S-IgA prevents contact of pathogens with mucosal surfaces by facilitating entrapment of pathogens in mucus followed by peristaltic or ciliary clearance (24, 48). In addition, IgA may directly block or sterically hinder the microbial attachment proteins that mediate epithelial attachment or may even intercept incoming pathogens within epithelial cell vesicular compartments (8, 9, 24, 28). The importance of S-IgA in protection against mucosal viral infections has been supported by studies in which protection was associated with the presence of specific IgA in secretions (for a review, see reference 34). On the other hand, there is evidence that S-IgA is not essential and that IgG alone can prevent mucosal infection (11, 39, 40, 56). The development of a transgenic mouse in which the IgA switch and constant regions are deleted has provided a valuable model in which T-cell function and production of other immunoglobulin isotypes are normal or elevated but IgA is absent from serum and secretions (22). Immunization-challenge experiments using this IgA knockout (IgA<sup>-/-</sup>) model have indicated that IgA is not necessary for protection against influenza virus infection of respiratory epithelium (29), herpes simplex virus infection of the vaginal

epithelium (44), *Helicobacter pylori* colonization of the gastric mucosa (6), or rotavirus infection of the intestinal epithelium (41).

The relative importance of S-IgA in protection against mucosal entry of other pathogens cannot be predicted from the above studies, however, because each microorganism has a preferred site of invasion and a distinct strategy for subverting epithelial barrier function and establishing mucosal infection. A striking example is the mouse pathogen reovirus that exploits the transepithelial transport activity of M cells to enter the Peyer's patch mucosa and initiate infection (63). After oral ingestion of reovirus type 1 Lang (T1L), the outer capsid of native virions is processed by proteases in the lumen of the intestine (5, 7), resulting in intermediate subviral particles (ISVPs) that adhere selectively to M-cell surfaces (2). Adherent viruses are transcytosed in vesicles to the intraepithelial M-cell pocket and the subepithelial tissue, and over the next 2 days, reovirus replicates in cells of the Peyer's patch mucosa (17, 42). In neonates, the infection then spreads systemically, but in adult mice the infection is usually limited to the mucosa, although viral antigens and/or antigen-sensitized cells later appear in the mesenteric lymph nodes and spleen (17). Infection of adult mice by reovirus T1L results in host immune responses that include specific serum IgG, S-IgA, and cytotoxic T lymphocytes (CTLs) (26, 27, 46, 58), and the infection is cleared within about 10 days (27). There is evidence that both CTLs and serum antibodies contribute to clearance of an established infection (4, 54). However, it is not known whether mice that have cleared an initial infection are protected against reinfection of Peyer's patches upon oral rechallenge and, if so, whether IgA is essential for protection.

In suckling mice, serum IgG alone was unable to prevent entry or early replication of reovirus in Peyer's patches. Reo-

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virus-specific, neutralizing IgG monoclonal antibodies (MAbs) passively transferred by intravenous injection failed to inhibit uptake and local replication of orally administered reovirus T1L in Peyer's patches, although they did prevent systemic spread (52, 53). In suckling mice orally challenged with reovirus type 3 Dearing (T3D), reovirus replication in the intestinal mucosa was prevented in pups that were suckled on orally immunized (but not subcutaneously immunized) dams (14). Rodent milk contains high levels of IgG that is transferred from the intestine into the neonatal circulation by receptor-mediated transcytosis (45), but in this case, protection was attributed to the reovirus-specific S-IgA antibodies in milk that were present only in the orally immunized dams. In the intestinal lumens of normal adult mice, there is abundant IgA but little IgG (21). Although this suggests that S-IgA would be required to prevent M-cell adherence and entry of reovirus in adults, the relative importance of S-IgA in protection of the intestinal mucosa against reovirus reinfection has not been directly tested. A complicating factor is that IgA as well as IgA-antigen complexes (but not IgG or IgM) selectively adheres to apical surfaces of M cells in adult mice (35, 59). Thus, the presence of specific IgA in the intestinal lumen during oral reovirus challenge could result in two very different outcomes: IgA-coated viral particles could be entrapped in mucus and cleared, or IgA could facilitate M-cell-mediated viral uptake and infection.

In this study, we sought to assess the role of IgA antibodies in protection against entry of reovirus (T1L) into Peyer's patches of adult mice. In an active immunization-rechallenge protocol, adult mice that had cleared an initial infection and produced both intestinal IgA and serum IgG antibodies directed against reovirus T1L outer capsid proteins were protected against reinfection of Peyer's patches upon oral rechallenge. When IgA knockout mice were subjected to the same protocol, they cleared the initial infection but their Peyer's patches became infected upon oral rechallenge, despite the presence of antiviral IgG in serum and elevated antiviral IgM in secretions. The results of this study indicate that S-IgA is a crucial component of mucosal protection against reovirus and that anti-reovirus IgA protects by preventing adherence of virus to M cells of the Peyer's patch epithelium.

#### MATERIALS AND METHODS

**Virus growth and purification.** Mouse L929 fibroblast cells (L cells) were grown in suspension culture in Joklik minimal essential medium (Irvine Scientific, Santa Ana, Calif.) containing 5% fetal calf serum (HyClone Laboratories, Logan, Utah), 2 mM glutamine, 100 U of penicillin/ml, and 100 µg of streptomycin/ml (all from Gibco BRL, Grand Island, N.Y.). Purified T1L virions were prepared using second-passage L cells infected with plaque-purified reovirus as previously described (16). Virus was released from infected cells by freezing-thawing and sonication, recovered from lysates through two Freon 113 (trichlorotrifluoroethane) extractions, and then purified by cesium chloride gradient centrifugation. The virus band was removed, dialyzed extensively against dialysis buffer (150 mM NaCl, 15 mM MgCl<sub>2</sub>, 10 mM Tris, pH 7.4) at 4°C, and stored at 4°C in dialysis buffer. The concentration of viral particles was calculated from protein concentration (16), and concentration of infectious virus was determined by plaque assay (53). The purity of viral preparations was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis on 10% polyacrylamide reducing gels (7).

**Mice.** Adult female BALB/c and C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, Mass.). IgA<sup>-/-</sup> (C57BL/6 × 129/Sv) mice were originally generated and described by Harriman and collaborators (22) and were generously provided to us by John Nedrud, Case Western Reserve University.

Animals were maintained in the animal resource facility at the Children's Hospital, and all animal procedures were conducted in strict compliance with the Guidelines for Animal Experimentation established by Harvard Medical School, the Children's Hospital, and the National Institutes of Health.

**Quantitation of viral entry into Peyer's patch tissue by plaque assay.** Mice were anesthetized by intraperitoneal administration of Avertin, 250 mg/kg of body weight (2,2,2-tribromoethanol; Aldrich, Milwaukee, Wis.), and sacrificed by cervical dislocation. Small intestines were removed and placed in incomplete Dulbecco's minimal essential medium (Gibco BRL) on ice. Peyer's patches were excised and collected in preweighed microcentrifuge tubes containing 1 ml of gelatin saline, pH 7 (per liter, 8 g of NaCl, 0.03 g of CaCl<sub>2</sub>, 0.17 g of MgCl<sub>2</sub>·6H<sub>2</sub>O, 1.2 g of H<sub>3</sub>BO<sub>3</sub>, 0.05 g of Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>·10H<sub>2</sub>O, 3.0 g of gelatin), with 2% Fungibact (Irvine Scientific). Tissue weight was determined, and Peyer's patches were disrupted by freezing-thawing twice, followed by probe sonication. Tissue plaque assays were done as previously described (56), and concentration of infectious virus in Peyer's patch tissue was expressed as PFU per milligram of tissue.

**Challenge-rechallenge assay.** For initial inoculations, mice were given  $2 \times 10^7$  PFU of reovirus in 500 µl of phosphate-buffered saline (PBS) intragastrically using a feeding needle. Naive mice were not intubated. On day 21, feces and serum were collected from all mice and subsets of animals from the reovirus-exposed and naive groups were orally challenged. Appropriate challenge doses, defined as doses that resulted in measurable infections in the Peyer's patches of all unprotected animals, were determined by pilot studies as  $2 \times 10^7$  PFU for BALB/c mice and  $5 \times 10^8$  PFU for C57BL/6 and IgA<sup>-/-</sup> (C57BL/6 × 129/Sv) mice. All groups of mice were sacrificed 8 h after rechallenge, and reovirus PFU per milligram of Peyer's patch tissue was determined as described above.

**Evaluation of reovirus-specific antibodies in secretions and serum.** Feces were collected from all mice on day 21 and placed in preweighed microcentrifuge tubes containing 1 ml of PBS containing 0.5% (wt/vol) nonfat dry milk and protease inhibitors (aprotinin, 1 µg/ml [Sigma]; leupeptin, 5 µg/ml [Sigma]; aminoethylbenzenesulfonyl fluoride, 48 µg/ml [Calbiochem, La Jolla, Calif.]; and bestatin, 1 µg/ml [Sigma]). Fecal pellets were disrupted by vortexing, and supernatants were obtained by centrifugation at maximum speed in an Eppendorf microcentrifuge for 20 min at 4°C. Aliquots of supernatants were stored at -20°C. For enzyme-linked immunosorbent assays (ELISAs), 96-well flat-bottomed plates (Nunc MaxiSorp, Roskilde, Denmark) were coated overnight with  $2 \times 10^{11}$  viral particles/ml in PBS at 4°C in a humidified chamber. Plates were washed in PBS containing 0.05% Tween (PBS-Tween), and nonspecific protein binding sites were blocked by addition of blocking buffer (PBS-Tween with 1% fetal calf serum). Serial twofold dilutions of serum and fecal supernatants in blocking buffer were applied in duplicate. Known concentrations of MAbs RB8 (anti-µ1c IgA) and 5C6 (anti-σ1 IgG) were used as standards for reovirus-specific IgA and IgG, respectively. A reovirus-specific IgM standard was not available, and so a preparation of pooled serum containing reovirus-specific IgM was assigned an arbitrary unit value and used as standard; concentrations in samples were expressed as ELISA units per milliliter. After washing in PBS-Tween, secondary biotinylated goat anti-mouse IgA, IgG, or IgM (Southern Biotechnology Associates, Birmingham, Ala.) was added at a 1:3,000 dilution in blocking buffer. Bound antibody was detected with a 1:5,000 dilution of streptavidin-horseradish peroxidase (Pierce, Rockford, Ill.) and the TMB one-component peroxidase-substrate detection system (Kirkegaard and Perry Laboratories, Gaithersburg, Md.). Plates were read at 650 nm in a SpectraMax 250 plate reader using the Softmax ELISA analysis program.

**Evaluation of total IgA, IgG, and IgM levels in serum and feces.** Fecal supernatants were prepared as described above. Ninety-six-well flat-bottomed plates (Nunc MaxiSorp) were coated with goat anti-mouse IgA (Southern Biotechnology Associates), goat anti-mouse IgG (Cappel, Durham, N.C.), or goat anti-mouse IgM (Cappel). Plates were washed in PBS-Tween and blocked as described above. Serial twofold dilutions of fecal supernatants or serum were applied in duplicate to the plates, along with standards. Standards were purified mouse monoclonal IgA (Southern Biotechnology Associates), purified mouse serum IgG (Sigma), and purified mouse monoclonal IgM (TEPC 183; Sigma). After washing with PBS-Tween, secondary antibodies were applied and detected as described above.

**Western blot analysis.** Gradient-purified reovirus was boiled in sample buffer (0.5 M Tris-Cl, 2% β2-mercaptoethanol, 0.1% bromophenol blue, 20% glycerol, 4% sodium dodecyl sulfate). Viral proteins were separated by electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose (Bio-Rad). Strips were blocked in PBS-5% fetal calf serum-0.1% Tween, incubated with individual fecal and serum samples, and then washed in PBS-0.05% Tween. Biotinylated goat anti-mouse IgA, IgG, or IgM (1:3,000; Southern Biotechnology Associates) was added, followed by incubation with streptavidin-horseradish peroxidase (1:

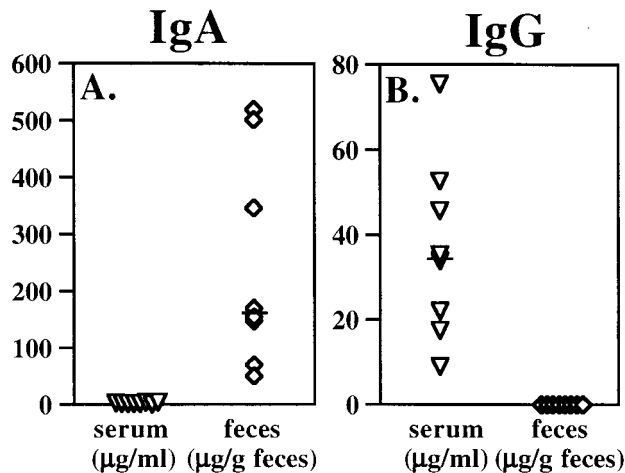


FIG. 1. Reovirus-specific antibodies in serum and intestinal secretions of eight adult BALB/c mice, 21 days after oral inoculation with reovirus T1L. Fecal antibodies were almost exclusively of the IgA isotype (A), while the serum response was dominated by IgG (B). Each symbol represents an individual mouse, and bars indicate medians.

500; Pierce). After washing, blots were developed with the Opti-4CN kit (Bio-Rad).

**MAbs and passive immunization protocol.** The reovirus-specific IgA and IgG MAbs used in this study were as follows. MAb 4A3 (IgG2b) is specific for reovirus outer capsid protein  $\mu 1c$ , 10G10 (IgG2a) is specific for the outer capsid protein  $\sigma 3$ , and 5C6 (IgG2a) is specific for the viral attachment protein  $\sigma 1$  (55). Two IgA MAbs were previously obtained by fusion of Peyer's patch cells after mucosal immunization of BALB/c mice: IgA RB3 is specific for the  $\sigma 3$  protein and IgA RB8 is specific for  $\mu 1c$ . Both were produced by cloned hybridoma cells as a mixture of monomers, dimers, and higher polymers (59). Hybridoma cells were grown in a Tecnomouse hollow-fiber apparatus (Integra Biosciences, Lowell, Mass.). Total IgA and IgG concentrations in Tecnomouse culture supernatants were measured by ELISA as previously described (21). BALB/c mice were used to assess the effects of orally administered IgA and IgG MAbs on viral entry. Unanesthetized mice were inoculated intragastrically with 500  $\mu$ l of PBS containing 50  $\mu$ g of reovirus-specific IgA or IgG MAb and  $2 \times 10^7$  PFU of reovirus T1L. Peyer's patch tissue was removed 8 h after oral inoculation, and virus was quantitated by plaque assay.

**Statistics.** The Statview 5.0.1 computer program (Abacus Concepts, Berkeley, Calif.) was used for all calculations and statistical analyses. Results were logarithmically transformed to obtain geometric means. Between-group comparisons were performed by unpaired two-tailed *t* test at the 99% confidence level. Results of all statistical analyses were considered significant only if *P* values were 0.01 or less.

## RESULTS

**Reovirus-specific antibodies in secretions and serum of orally inoculated adult BALB/c mice.** In pilot experiments,  $2 \times 10^7$  PFU of T1L was identified as an oral challenge dose that consistently resulted in the presence of virus in Peyer's patches of adult BALB/c mice, as detected by viral plaque assays of Peyer's patch tissue at 8 h after feeding. Peyer's patch PFU measured at this time interval represents virus that has entered via M cells and is undergoing early local replication (17, 23). Two groups (eight mice per group) were orally inoculated on day 0 with reovirus, while two groups of control naive mice received no virus. On day 21, about 10 days after the viral infection had been cleared, samples of serum and feces were collected from all 32 mice. A group of eight reovirus-exposed mice and a group of eight naive controls were then orally challenged with reovirus, and 8 h later, Peyer's patches were

collected and viral entry was evaluated by plaque assay. Reovirus-specific IgA, IgG, and IgM antibodies present in serum and feces at the time of challenge were measured by ELISA. In all mice exposed to virus on day 0, reovirus-specific IgA antibodies were present in feces at day 21 (Fig. 1). Reovirus-specific IgG was undetectable in fecal supernatants of these mice, but a specific serum IgG response was present (Fig. 1). To determine the antigen specificity of these humoral responses, fecal supernatants and serum samples were applied to Western blots of reovirus proteins (Fig. 2). IgA immunoglobulins in fecal samples from naive control animals bound nonspecifically to multiple reovirus protein bands including  $\sigma 3$  and  $\mu 1c$  (Fig. 2, lanes 7 to 9), so that the extent to which fecal IgA in reovirus-exposed mice specifically recognized these two outer capsid proteins could not be determined. Fecal IgA from naive mice did not bind to the  $\sigma 1$  band, however, and in fecal samples from mice previously exposed to virus, IgA antibodies specific for  $\sigma 1$  were consistently present (Fig. 2, lanes 10 to 12). Western blots also revealed that the serum IgG response to reovirus was focused primarily on the  $\sigma 1$  protein (Fig. 2, lanes 4 to 6). Thus, all mice that were inoculated with reovirus on day 0 had anti- $\sigma 1$  IgA antibodies in intestinal secretions and anti- $\sigma 1$  IgG antibodies in serum at the time of oral challenge on day 21.

**Previous mucosal infection and clearance of reovirus protects adult mice against subsequent mucosal challenge.** A second set of BALB/c mice were then orally inoculated with reovirus (or not inoculated) on day 0 and rechallenged (or not) on day 21. Peyer's patches were collected 8 h after challenge for plaque assay. Seven mice inoculated on day 0 that were not rechallenged had cleared the infection from the Peyer's patch

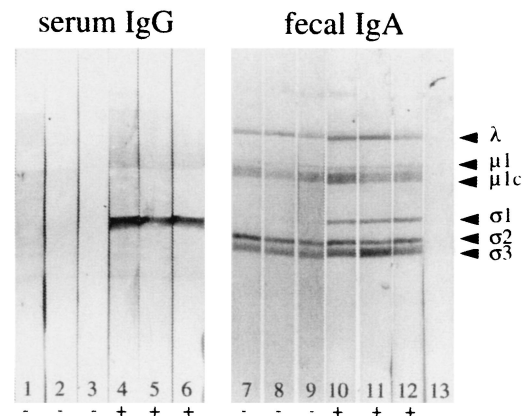


FIG. 2. Western blots of reovirus proteins showing anti- $\sigma 1$  serum IgG and fecal IgA antibodies in BALB/c mice, 21 days after oral reovirus inoculation. Serum and fecal supernatants from six naive control mice and eight reovirus-inoculated mice were tested; three representative samples from each group are shown. Lanes 1 to 3, sera from naive control mice showed no antireovirus immunoreactivity. Lanes 4 to 6, sera from reovirus-exposed mice contained IgG antibodies directed primarily against the  $\sigma 1$  protein. Lanes 7 to 9, IgA in fecal extracts from naive control mice bound nonspecifically to reovirus proteins including  $\sigma 3$  and  $\mu 1c$ , but not to  $\sigma 1$ . Lanes 10 to 12, fecal extracts from reovirus-exposed mice showed the presence of anti- $\sigma 1$  IgA antibodies. IgA antibodies specific for other viral proteins could not be visualized because of nonspecific binding. Lane 13, control strip exposed to secondary antibody alone.



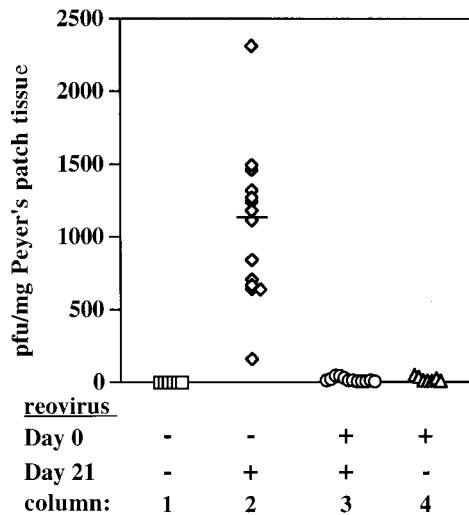


FIG. 3. Reovirus in Peyer's patches of naive and reovirus-immunized BALB/c mice, 8 h after mice were rechallenged (or not) on day 21. Column 1, naive mice had no infectious virus in their Peyer's patches ( $n = 6$ ). Column 2, naive mice orally challenged with reovirus ( $2 \times 10^7$  PFU) had infectious virus in Peyer's patches 8 h after challenge ( $n = 14$ ). The bar represents the median PFU per milligram of tissue. Column 3, mice orally inoculated with reovirus on day 0 were completely protected against oral reovirus rechallenge on day 21 ( $n = 12$ ). Column 4, mice orally inoculated with reovirus on day 0 had cleared virus from their Peyer's patches by day 21 ( $n = 7$ ). Each symbol represents an individual mouse.

mucosa by day 21 (Fig. 3, column 4). All 14 of the naive mice that were orally challenged with reovirus on day 21 had infectious virus in their Peyer's patches 8 h later (Fig. 3, column 2). However, 12 mice that had previously cleared a reovirus infection showed no evidence of reinfection (Fig. 3, column 3). Whether virus was prevented from entering the Peyer's patch by secreted IgA antibodies or neutralized within the patch by serum IgG antibodies or virus-specific CTLs could not be determined in these normal mice. To address this issue, we repeated the above experiment using  $IgA^{-/-}$  mice.

**Preexposure to reovirus did not prevent mucosal infection upon oral challenge with reovirus in  $IgA^{-/-}$  mice.** To determine if reovirus-immunized  $IgA^{-/-}$  mice would be protected against viral entry upon oral rechallenge, groups (five mice per group) of  $IgA^{-/-}$  mice and C57BL/6 controls were inoculated (or not) on day 0 and challenged (or not) 21 days later. Although C57BL/6 mice were not an optimal match, they were preferable to BALB/c mice as controls for the C57BL/6  $\times$  129/Sv  $IgA^{-/-}$  mice. Feces and serum samples were collected on the day of challenge for subsequent ELISA analysis as described below. Determination of PFU in Peyer's patch tissue taken on day 21 from immunized mice that were not rechallenged showed that virus had been cleared from the intestines of both C57BL/6 controls and  $IgA^{-/-}$  mice by this time (Fig. 4, columns 4 and 7). Comparison of Peyer's patch tissues from naive  $IgA^{-/-}$  and naive wild-type mice that were challenged with reovirus on day 21 showed that both were infected at 8 h after challenge, and the difference between these two groups was not significant (Fig. 4, columns 2 and 5). On average, however, the mucosa of  $IgA^{-/-}$  mice contained higher

amounts of virus, suggesting that IgA in secretions of wild-type mice may have provided some nonspecific protection. Peyer's patch tissues from previously exposed, wild-type C57BL/6 mice were completely virus free 8 h after rechallenge (Fig. 4, column 3), confirming the results in BALB/c mice. In contrast, Peyer's patch tissues from previously exposed  $IgA^{-/-}$  mice consistently contained infectious virus (Fig. 4, column 6), although in significantly lower quantities than  $IgA^{-/-}$  mice that had not been previously exposed (Fig. 4, column 5).

**Total and reovirus-specific antibodies in secretions and serum of orally inoculated  $IgA$  knockout mice.** Humoral immune responses to reovirus in  $IgA^{-/-}$  and control mice were analyzed in serum and fecal samples collected 21 days after oral inoculation in two separate sets of mice. One set, consisting of four groups (wild type and  $IgA^{-/-}$ , inoculated and naive), was used to measure levels of total immunoglobulins and reovirus-specific antibodies; these mice were not rechallenged. Reovirus-specific antibodies were measured in the second set described above (five mice per group), which were inoculated (or not) on day 0 and rechallenged (or not) on day 21 as shown in Fig. 4. Levels of reovirus-specific antibodies in the two sets of mice were comparable. ELISA analysis confirmed that there

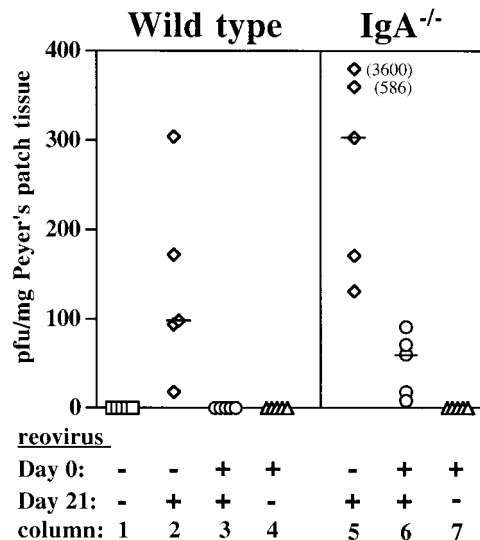


FIG. 4. Viral entry into Peyer's patches of naive and reovirus-exposed C57BL/6 (wild-type) and  $IgA^{-/-}$  mice, 8 h after oral rechallenge with  $5 \times 10^8$  PFU of reovirus at day 21. Column 1, Peyer's patches of naive wild-type mice contained no virus. Column 2, naive wild-type mice orally challenged with reovirus had infectious virus in Peyer's patches 8 h after challenge. Column 3, previously inoculated wild-type mice were completely protected against oral reovirus rechallenge on day 21. Column 4, wild-type mice orally inoculated with reovirus on day 0 had cleared virus from their Peyer's patches by day 21. Column 5, naive  $IgA^{-/-}$  mice orally challenged with reovirus had infectious virus in Peyer's patches 8 h after challenge. The median PFU per milligram of tissue was higher than in comparable wild-type mice shown in column 2, but the difference was not significant ( $P = 0.086$ ). Column 6, previously inoculated  $IgA^{-/-}$  mice were not protected against oral reovirus rechallenge on day 21, although levels of infectious virus in Peyer's patches were significantly lower than those in naive  $IgA^{-/-}$  challenged with reovirus ( $P = 0.01$ ). Column 7,  $IgA^{-/-}$  mice orally inoculated with reovirus on day 0 had cleared virus from their Peyer's patches by day 21. Each symbol represents an individual mouse, and bars indicate the medians for each group.

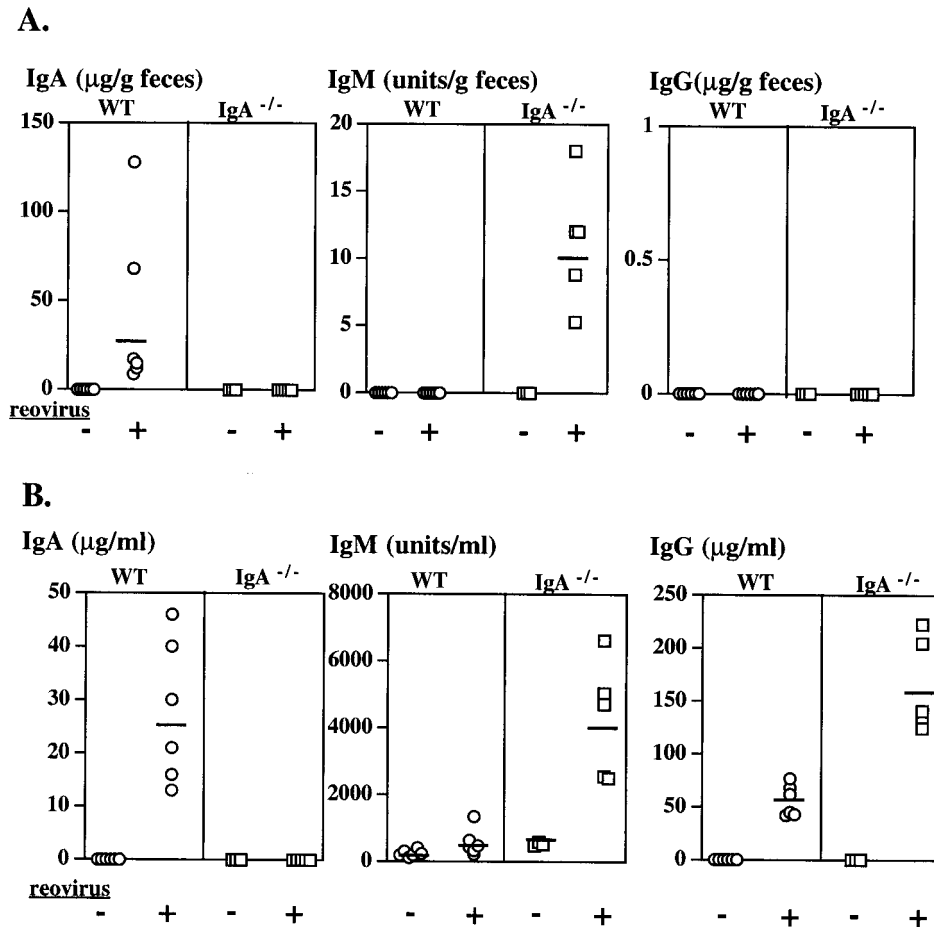


FIG. 5. Reovirus-specific IgA, IgM, and IgG levels in feces and serum of wild-type (WT) and  $IgA^{-/-}$  mice, either naive (-) or reovirus exposed (+), on day 21 after oral inoculation. Each symbol represents an individual mouse, and bars indicate geometric means for each group. IgM levels are expressed as arbitrary ELISA units based on a standard pooled serum preparation from reovirus-immunized mice. IgA and IgG levels are measured as micrograms per milliliter of serum or micrograms per gram of feces, determined using antireovirus monoclonal IgA (monomer-dimer mixture) or IgG as standard. (A) Reovirus-specific IgA, IgM, and IgG in feces. No specific IgA was detected in  $IgA^{-/-}$  mice, as expected. Specific IgM was detected, but levels were very low (mean, 10.4 U/g) compared to serum IgM levels (mean, 3,984 U/ml, shown in panel B). No reovirus-specific IgG was detected in feces of WT or  $IgA^{-/-}$  mice. (B) Reovirus-specific IgA, IgM, and IgG in serum. Specific serum IgA levels in reovirus-inoculated C57BL/6 mice were higher (mean, 25  $\mu$ g/ml) than in comparable BALB/c mice shown in Fig. 1.  $IgA^{-/-}$  mice had significantly higher levels of specific serum IgM and IgG than did WT mice (for both,  $P \leq 0.001$ ).

was no detectable IgA in intestinal secretions or serum of  $IgA^{-/-}$  mice as previously documented (22), but there were high levels of IgA (mean, 940  $\mu$ g/ml) in feces of C57BL/6 controls. We also confirmed that mean total IgM levels in fecal samples were low but were significantly higher in  $IgA^{-/-}$  mice (3.1  $\mu$ g/g of feces) than in wild-type mice (1.0  $\mu$ g/g of feces) ( $P \leq 0.0001$ ). Total IgG levels in feces of  $IgA^{-/-}$  and wild-type mice were low and comparable, ranging from 1 to 8.5  $\mu$ g of IgG/g of feces. In the serum of  $IgA^{-/-}$  mice, total IgM levels were significantly higher (mean, 1,040  $\mu$ g/ml) than in C57BL/6 controls (mean, 363  $\mu$ g/ml) ( $P = 0.0027$ ). Total IgG levels in  $IgA^{-/-}$  mice (mean, 12.04 mg/ml) were also significantly higher than in wild-type controls (mean, 2.28 mg/ml) ( $P = 0.0079$ ), confirming the original description of these mice (22).

Reovirus-specific antibodies in fecal and serum samples are shown in Fig. 5. Antireovirus IgA antibodies were present in feces of wild-type C57BL/6 mice (although at lower levels than in BALB/c mice) but were not present in the  $IgA^{-/-}$  mice as

expected (Fig. 5A). However, reovirus-specific IgM was detected in the feces of  $IgA^{-/-}$  mice, whereas it was undetectable in wild-type mice. In neither group were reovirus-specific IgG antibodies detected in fecal supernatants. As expected, reovirus-specific IgA was present in sera of wild-type mice but was undetectable in  $IgA^{-/-}$  mice (Fig. 5B). Levels of reovirus-specific serum IgM mirrored total IgM levels:  $IgA^{-/-}$  mice (but not wild-type controls) produced detectable levels of specific IgM. Reovirus-specific serum IgG levels were also higher in  $IgA^{-/-}$  mice (mean, 161  $\mu$ g/ml) than in wild-type mice (mean, 54  $\mu$ g/ml), and this difference was significant. Taken together, the viral plaque assays and ELISA data showed that protection of previously exposed C57BL/6 mice against Peyer's patch infection was associated with high levels of specific and total IgA in secretions, and the absence of IgA in secretions of  $IgA^{-/-}$  mice resulted in an inability to prevent viral entry and replication. Although elevated IgM antibodies in secretions and high levels of IgG and IgM antibodies in serum of the

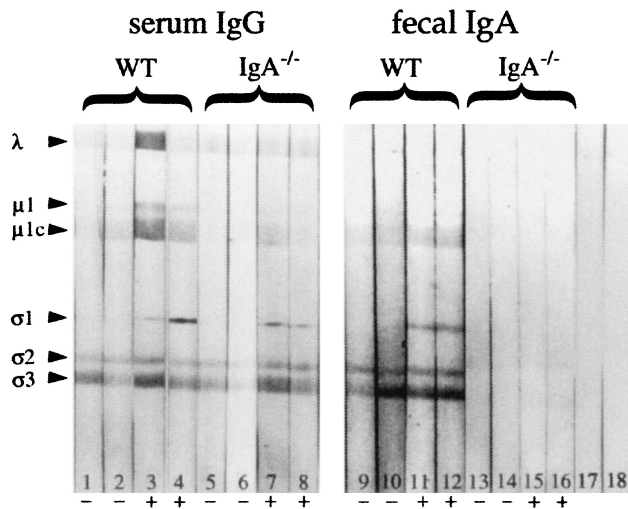


FIG. 6. Western blot analysis of serum and fecal supernatants from C57BL/6 (wild-type [WT]) and  $IgA^{-/-}$  mice collected 21 days after oral reovirus inoculation. Serum and fecal supernatants of two representative mice from each group are shown. Lanes 1 and 2, sera from naive control mice showed no anti- $\sigma 1$  immunoreactivity although nonspecific binding of IgG to other reovirus proteins was seen. Lanes 3 and 4, anti- $\sigma 1$  IgG antibodies were consistently present in the sera of reovirus-exposed WT mice. IgG binding to other proteins was variable. Lanes 5 and 6, sera from naive  $IgA^{-/-}$  mice showed some nonspecific IgG binding to some reovirus proteins but not to  $\sigma 1$ . Lanes 7 and 8, sera from  $IgA^{-/-}$  mice orally inoculated with reovirus contained anti- $\sigma 1$  IgG antibodies. Lanes 9 and 10, fecal supernatants of naive control WT mice did not contain detectable anti- $\sigma 1$  antibodies, although nonspecific IgA binding to other reovirus proteins was observed. Lanes 11 and 12, anti- $\sigma 1$  IgA antibodies were detected in fecal supernatants of reovirus-exposed WT mice. Lanes 13 to 16, no reovirus-specific or nonspecific IgA was detected in fecal supernatants of naive or reovirus-exposed  $IgA^{-/-}$  mice. Lanes 17 and 18, control strips exposed to secondary goat anti-mouse IgG and to IgA, respectively.

$IgA^{-/-}$  mice appeared to have provided partial protection against reovirus challenge, the Peyer's patches nevertheless became infected.

Western blot analysis of fecal supernatants from reovirus-inoculated mice confirmed that  $IgA^{-/-}$  mice had no detectable reovirus-specific IgA antibodies in secretions (Fig. 6, lanes 13 to 16) while wild-type C57BL/6 mice had IgA antibodies specific for  $\sigma 1$  (Fig. 6, lanes 11 and 12). Responses to  $\mu 1c$  and  $\sigma 3$  could not be assessed due to nonspecific binding of fecal IgA to these proteins. Sera of both wild-type and  $IgA^{-/-}$  mice contained specific IgG antibodies directed against  $\sigma 1$  (Fig. 6, lanes 3 and 4 and lanes 7 and 8), and some IgG reactivity against  $\mu 1c$  and other viral proteins was inconsistently observed. No specific IgM in sera or secretions of control or knockout mice was detected on Western blots (data not shown).

**Effects of antireovirus MAbs on virus entry into Peyer's patch mucosa.** In the immunized BALB/c and C57BL/6 wild-type mice described above, protection against rechallenge was consistently associated with the presence of fecal IgA antibodies directed against the  $\sigma 1$  attachment protein. Whether antibodies against  $\mu 1c$  and  $\sigma 3$  proteins were also present could not be determined because of nonspecific IgA binding on Western blots, but we considered their presence likely because IgA lymphoblasts recovered from mouse Peyer's patches of reovi-

rus-exposed mice were previously shown to be specific for these antigens (59). The outer capsid protein responsible for reovirus adherence to M cells has not been identified, but previous studies using other enteric pathogens have shown that IgAs against abundant microbial surface antigens can protect the mucosa by immune exclusion, even if the antigen is not directly involved in host cell adherence (9, 33, 61). Monoclonal IgA antibodies specific for the abundant outer capsid proteins  $\mu 1c$  and  $\sigma 3$  (but not the viral attachment protein  $\sigma 1$ ) were available. We therefore sought to determine whether specific luminal IgA MAbs directed against the viral outer capsid may protect against infection of mouse Peyer's patches by preventing M-cell contact or may allow viral entry by mediating adherence to M cells. Groups of mice were orally challenged with reovirus mixed with antireovirus IgA MAbs (at a ratio of about  $6 \times 10^4$  IgA dimers or  $12 \times 10^4$  IgA monomers per viral particle), and viral entry in Peyer's patch tissue was measured 8 h later. Other groups received IgG MAbs against  $\mu 1c$  and  $\sigma 3$  for comparison, and control mice received virus with no antibody. Neither the IgG nor the IgA MAbs directed against  $\sigma 3$  prevented reovirus entry (Fig. 7A). This is consistent with the fact that this protein is removed by proteases in the intestinal lumen (7). The  $\mu 1c$  protein is not removed, however, and thus, anti- $\mu 1c$  IgA MAbs would be expected to remain associated with viral particles. However, neither the IgA nor the IgG MAbs directed against  $\mu 1c$  reduced viral entry or early replication (Fig. 7B). Thus, the IgA MAbs apparently failed to prevent viral contact with the mucosa by immune exclusion and failed to prevent M-cell-mediated reovirus uptake.

In the absence of an IgA MAb specific for the viral attachment protein  $\sigma 1$ , we tested an available anti-reovirus T1L IgG MAb, 5C6 (55), which recognizes the head region of the  $\sigma 1$  attachment protein (10). MAb 5C6 was neutralizing in cell culture assays and protected neonatal mice against intracranial injection of reovirus T1L (53). When mixed with virus (at a ratio of about  $1.2 \times 10^5$  molecules of IgG per viral particle) and fed to mice, this MAb consistently blocked viral infection of Peyer's patch mucosa (Fig. 7C). Although significant amounts of IgG are not normally present in mouse intestinal secretions, this result served to validate the passive feeding protocol by showing that MAbs remained associated with virus in the intestinal lumen. More importantly, it indicated that specific blocking of the viral attachment protein  $\sigma 1$  prevented M-cell-mediated entry whereas blocking of other outer capsid proteins did not.

## DISCUSSION

The results of this study show that antireovirus IgA secreted into the intestines of immunized mice plays a crucial role in protection against mucosal reinfection. This is consistent with the well-documented role of S-IgA in preventing pathogen entry into mucosal tissues (24, 32, 48). From studies of a variety of viral pathogens including reovirus and rotavirus, it is generally agreed that cell-mediated immunity plays a major role in clearing viral infections but that antibodies are essential for prevention of infection (18, 19, 26, 27, 30, 31). The relative importance of S-IgA and IgG antibodies in mucosal protection is still controversial, however. Studies in which antireovirus IgG was induced in serum by immunization or passively deliv-

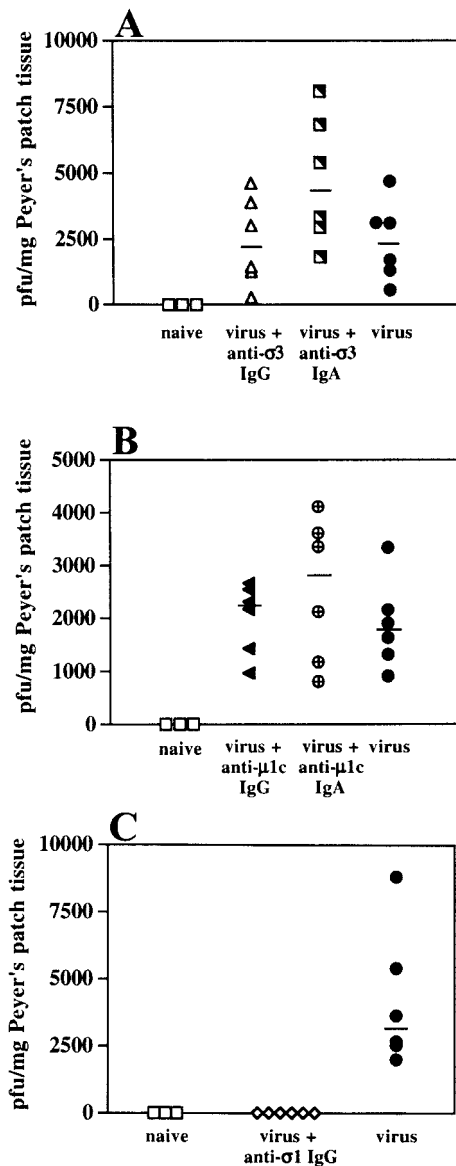


FIG. 7. Effects of antireovirus MABs on viral entry into Peyer's patches in BALB/c mice. Aliquots of reovirus ( $2 \times 10^7$  PFU) were mixed with 50  $\mu$ g of MAB and inoculated intragastrically into groups of six mice. Naive mice ( $n = 3$ ) were not inoculated, and control mice ( $n = 6$ ) received virus only. After 8 h, Peyer's patches were removed and viral entry was assessed by plaque assay. Each symbol represents an individual mouse, and bars indicate median PFU. (A) Neither anti- $\sigma 3$  IgG nor anti- $\sigma 3$  IgA MAB affected viral entry. Although viral titers in the patches were somewhat higher in the presence of the anti- $\sigma 3$  IgA MAB than with the anti- $\sigma 3$  IgG MAB in the experiment shown, this difference was not significant and was not observed when the experiment was repeated (data not shown). (B) Neither anti- $\mu 1c$  IgG nor anti- $\mu 1c$  IgA MAB affected viral entry. (C) Anti- $\sigma 1$  IgG MAB 5C6 prevented viral infection of Peyer's patches in all mice tested ( $n = 6$ ).

ered onto mucosal surfaces indicated that IgG alone can be sufficient for protection (13, 39, 40), and this has been supported by recent studies using IgA knockout mice (41). In addition, wild-type and IgA<sup>-/-</sup> mice immunized with influenza virus subunit vaccines along with adjuvants (either cholera

holotoxin and B subunit or interleukin-12) showed equivalent levels of protection against pulmonary influenza virus challenge, and protection was attributed to the presence of specific serum IgG in the IgA<sup>-/-</sup> mice that presumably entered the lungs and airways (3, 29, 57).

It is not surprising that IgG can be sufficient to protect the respiratory tract where significant amounts of IgG, transudated from serum or produced locally, are normally present in secretions (34). More surprising is the report that immunized IgA<sup>-/-</sup> mice were protected against oral rotavirus challenge (41), since IgG levels in small intestinal secretions of mice are normally very low (21). Indeed, IgG levels in intestinal secretions of the normal mice that were rotavirus immunized and protected were low as expected, but the immunized IgA<sup>-/-</sup> mice (which were also protected) had elevated antiviral IgG levels in intestinal secretions (41). This suggests that, in the absence of IgA, rotavirus infection of villus epithelial cells caused a defect in epithelial barrier function, perhaps similar to that observed in rotavirus-infected epithelial monolayers (38), which persisted even after the initial infection had been cleared. In addition, serum IgG that diffuses freely from fenestrated villus capillaries and percolates between villus epithelial cells (1) might have restricted rotavirus spread within the epithelium. In contrast, the reovirus-immunized IgA knockout mice in our study did not have elevated antiviral IgG in intestinal secretions and were not protected against reinfection despite high levels of antireovirus IgG in serum.

This apparent discrepancy may reflect the fact that reovirus, unlike rotavirus, initiates infection only in the Peyer's patches (17, 37). Although reovirus-specific serum IgG antibodies against the viral attachment protein could theoretically prevent initial target cell infection and cell-to-cell spread of reovirus in the Peyer's patch mucosa (60), there is evidence that serum IgG diffuses poorly into Peyer's patch mucosa where capillaries are nonfenestrated (1). Indeed, neutralizing antireovirus IgG administered systemically failed to prevent viral entry or early replication in Peyer's patches of suckling mice (51, 52). Similarly, serum IgG failed to prevent the early stage of Peyer's patch infection in the IgA<sup>-/-</sup> adult mice in this study. The presence of antireovirus IgM was not sufficient for protection against reinfection in our experiments, although it has been suggested that secretory IgM might compensate for IgA in individuals with IgA deficiency (15). It is important to note that the levels of virus in the Peyer's patches of our immunized IgA<sup>-/-</sup> mice were lower than in unimmunized IgA<sup>-/-</sup> mice after oral reovirus challenge, suggesting that antiviral serum IgG (and possibly secretory IgM) did provide some protection against entry or early viral replication. However, in the absence of IgA it was not sufficient to completely prevent mucosal infection.

When naive mice that had no reovirus-specific antibodies were orally challenged with reovirus, the IgA<sup>-/-</sup> mice on average had higher viral titers in their Peyer's patches than did wild-type mice. This is not likely to be due to impaired cell-mediated immunity. Although a recent report suggested that the absence of IgA results in defective T-cell help (3), others had shown that T-cell functions are normal in the IgA<sup>-/-</sup> mice (22, 29). It is possible that C57BL/6 mice have more innate protective factors in the intestine than do C57BL/6  $\times$  129/Sv mice. However, we consider it likely that the presence of S-IgA



in the wild-type mice provided some degree of nonspecific mucosal protection against reovirus. Nonspecific IgA protection has been observed in studies of urinary tract infections with *Escherichia coli*, where protection proved to be due to interaction of bacterial lectin-like attachment proteins with IgA oligosaccharides (50, 62). Fecal IgA interacted nonspecifically with some reovirus proteins on Western blots, but whether such interactions occurred with intact viral particles in vivo is not known. Alternatively, the endogenous IgA that accumulates on M-cell surfaces of normal mice may have provided nonspecific protection by sterically hindering access of viral particles to the relevant M-cell surface binding sites. In any case, only the mice that had specific antireovirus IgA in intestinal secretions were completely protected against infection of Peyer's patch mucosa.

In mice previously exposed to reovirus, the Peyer's patch tissue presumably contained virus-specific CTLs. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells have been shown to be involved in protection against systemic reovirus disease (55), and enteric reovirus infection induced reovirus-specific CTLs in Peyer's patch tissue after intestinal priming (26). However, cell-mediated immunity would come into play only after the virus has entered the Peyer's patch and succeeded in infecting target cells. Indeed, there is evidence that CTLs alone are unable to prevent early proliferation in the mucosa. In studies using SCID mice, adoptive transfer of reovirus-immune, B-cell-depleted spleen cells prior to oral challenge did not prevent initial entry or replication of virus in intestinal tissue (4). Reovirus-specific CTLs would thus not be expected to prevent the initial infection of Peyer's patch target cells that was measured in our immunized IgA<sup>-/-</sup> mice at 8 h after challenge, although they could eventually have cleared infected cells and limited the infection.

In the process of establishing a mucosal infection in the Peyer's patches of naive mice, reovirus survives passage through the protease-rich environment of the intestinal lumen and then exploits specific binding sites exposed on M-cell surfaces. Enzymes in the intestinal lumen cleave off the reovirus  $\sigma 3$  outer capsid protein, which exposes the putative fusion protein  $\mu 1c$  and allows the viral attachment protein  $\sigma 1$  to extend from the viral surface (16, 20, 36). On the epithelial surface, the virus adheres to specific oligosaccharides on apical membranes of M cells containing  $\alpha(2-3)$ -linked sialic acid (K. J. Silvey et al., submitted for publication), which results in rapid vesicular transport across the epithelial barrier. In the small intestinal secretions of normal adult mice, the only immune effector available is S-IgA. S-IgA diffuses freely through mucus gels (12), but when complexed with antigen, its avidity for mucins increases (49), and this appears to be the basis for its ability to intercept pathogens and prevent mucosal contact by immune exclusion. Secretion of monoclonal IgA antibodies directed against microbial surface antigens that were nonneutralizing in cell culture systems has been shown to protect the small intestines of mice against bacterial and viral pathogens (9, 33, 47, 61). On this basis, we assume that immune exclusion played a role in the protection against reovirus challenge observed in our immunized wild-type mice. Studies using IgA or IgM MAbs against rotavirus (9), Sendai virus (28), and human immunodeficiency virus (8) have suggested an additional mechanism of protection in which secretory antibodies being exported by receptor-mediated transepithelial transport inter-

## Reovirus, antibodies and M cells

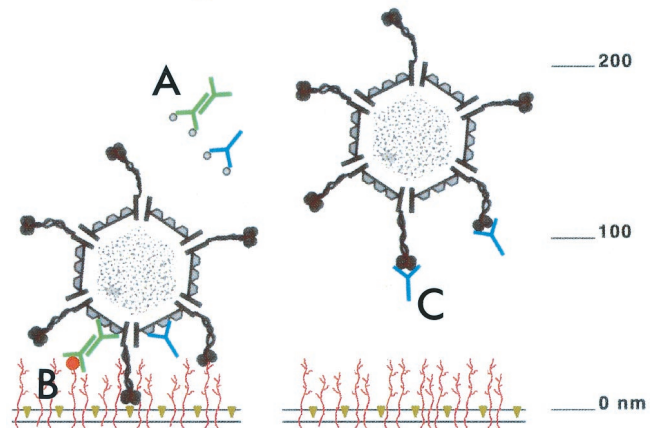


FIG. 8. Cartoon summarizing possible interactions of MAbs with reovirus in the passive protection experiments. The M-cell plasma membrane with integral glycolipids (gold) and glycoproteins (red), along with reovirus ISVPs (adapted from reference 37), is drawn approximately to scale (note nanometer scale at right). (A) Neither IgG (blue) nor dimeric IgA (green) directed against the  $\sigma 3$  outer capsid protein prevented M-cell attachment and Peyer's patch infection, presumably because  $\sigma 3$  is cleaved off by digestive proteases. (B) Anti- $\mu 1c$  IgG (blue) or dimeric IgA (green) may have failed to prevent reovirus attachment via the extended  $\sigma 1$  attachment protein. Thus, reovirus coated with anti- $\mu 1c$  IgA could have adhered to M cells via either  $\sigma 1$  or IgA. (The red dot represents a putative IgA receptor on the M cell.) (C) Anti- $\sigma 1$  IgG may have prevented entry and infection by blocking the interaction of the  $\sigma 1$  viral attachment protein with M-cell receptors.

cept incoming viruses within intracellular compartments of epithelial cells. It should be noted that anti- $\sigma 1$  IgA could not protect against reovirus by this mechanism because reovirus enters via M cells in the follicle-associated epithelium where IgA export does not occur (43).

The passive protection results in which IgAs against virus major outer capsid proteins did not prevent uptake and infection appear inconsistent with the protection associated with IgA secretion in immunized normal mice. However, it is important to note that our passive feeding protocol involved loading large amounts of virus and antibodies into the intestinal lumen. This may have resulted in considerable contact of virus-antibody complexes with the epithelium and M cells. Previous studies demonstrated that IgA adheres selectively to the apical membranes of M cells in mice and that adherent IgA-coated particles are transported across the epithelial barrier (59). M-cell adherence of IgA-coated reovirus could explain the fact that neither of the nonneutralizing IgA MAbs, which were directed against the outer capsid proteins  $\sigma 3$  and  $\mu 1c$ , prevented viral entry. If the  $\sigma 3$  protein along with associated IgA antibodies was cleaved off in the lumen by intestinal proteases, the virus could have adhered to M cells via the  $\sigma 1$  adhesin. If the anti- $\sigma 3$  IgA inhibited cleavage of  $\sigma 3$ , it is possible that the IgA itself could have mediated M-cell adherence. We have assumed, but not proven, that the anti- $\mu 1c$  antibodies could remain associated with the virus in the intestine because  $\mu 1c$  cleavage products are not lost from the viral surface and our anti- $\mu 1c$  IgA recognizes ISVPs (59). Anti- $\mu 1c$  IgA on the



viral surface may not have prevented interaction of the  $\sigma 1$  attachment protein with M cells, because the length of dimeric IgA is not thought to exceed 30 to 35 nm, whereas the extended  $\sigma 1$  is at least 40 nm long (16, 25) (Fig. 8). The fact that actively immunized, normal mice that secreted IgA against the viral attachment protein  $\sigma 1$  were protected, together with the observation that the anti- $\sigma 1$  IgG MAbs 5C6 was the only antibody that completely prevented Peyer's patch infection, suggested that IgA antibodies directed against  $\sigma 1$  might have played a particularly important role in protection of the immunized mice. Whether anti- $\sigma 1$  IgA antibodies can protect against viral entry by preventing M-cell adherence in spite of the potential IgA-M-cell interaction is unknown. This issue must be resolved in future studies when appropriate IgA MAbs directed against the  $\sigma 1$  viral attachment protein become available.

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