Protective Immunoglobulin A and G Antibodies Bind to Overlapping Intersubunit Epitopes in the Head Domain of Type 1 Reovirus Adhesin $\sigma 1$

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Nonfusogenic mammalian orthoreovirus (reovirus) is an enteric pathogen of mice and a useful model for studies of how an enteric virus crosses the mucosal barrier of its host and is subject to control by the mucosal immune system. We recently generated and characterized a new murine immunoglobulin A (IgA)-class monoclonal antibody (MAb), 1E1, that binds to the adhesin fiber, $\sigma 1$, of reovirus type 1 Lang (T1L) and thereby neutralizes the infectivity of that strain in cell culture. 1E1 is produced in hybridoma cultures as a mixture of monomers, dimers, and higher polymers and is protective against peroral challenges with T1L either when the MAb is passively administered or when it is secreted into the intestines of mice bearing subcutaneous hybridoma tumors. In the present study, selection and analysis of mutants resistant to neutralization by 1E1 identified the region of T1L $\sigma 1$ to which the MAb binds. The region bound by a previously characterized type 1 $\sigma 1$ -specific neutralizing IgG MAb, 5C6, was identified in the same way. Each of the 15 mutants isolated and analyzed was found to be much less sensitive to neutralization by either 1E1 or 5C6, suggesting the two MAbs bind to largely overlapping regions of $\sigma 1$. The tested mutants retained the capacity to recognize specific glycoconjugate receptors on rabbit M cells and cultured epithelial cells, even though viral binding to epithelial cells was inhibited by both MAbs. S1 sequence determinations for 12 of the mutants identified $\sigma 1$ mutations at four positions between residues 415 and 447, which contribute to forming the receptor-binding head domain. When aligned with the $\sigma 1$ sequence of reovirus type 3 Dearing (T3D) and mapped onto the previously reported crystal structure of the T3D $\sigma 1$ trimer, the four positions cluster on the side of the $\sigma 1$ head, across the interface between two subunits. Three such interface-spanning epitopes are thus present per $\sigma 1$ trimer and require the intact quaternary structure of the head domain for MAb binding. Identification of these intersubunit epitopes on $\sigma 1$ opens the way for further studies of the mechanisms of antibody-based neutralization and protection with type 1 reoviruses.

Antibodies are key elements in protective immunity against most viruses (reviewed in references 7, 27, 28, 45, and 59). Both antibodies in serum (primarily immunoglobulin G [IgG]) and antibodies secreted onto mucosal surfaces (primarily dimeric or polymeric IgA) (27, 59) make important contributions to protection from viruses that invade via mucosal routes. Serum IgG within mucosal tissues may protect by the same mechanisms as during neutralization of viruses in cell culture (e.g., by interference with receptor binding) (27, 28, 45, 59), but other serum and tissue factors that recognize virus-bound IgG (e.g., complement and phagocytes) participate as well (59). On mucosal surfaces, secretory IgA antibodies are thought to provide a first line of protection by facilitating entrapment of viral particles in mucus, blocking viral adherence to epithelial cell surfaces, and/or preventing viral entry across the epithelial barrier (38).

Reoviruses provide useful models for studies of many aspects of viral pathogenesis and host immunity (reviewed in reference 50). Type 1 and type 3 reoviruses, including the proto-

type strain type 1 Lang (T1L), adhere selectively to apical surfaces of M cells in the intestinal epithelium associated with Peyer's patches in adult mice and exploit the transepithelial transport activity of these cells (22, 60). M-cell adherence is a prerequisite for reovirus T1L infection in the adult intestine since it is required for initial entry of this virus into the intestinal mucosa (1, 22, 24, 61; reviewed in reference 34). We have focused our recent studies on the respective roles of secretory IgA and serum IgG in protecting the intestinal mucosa from reovirus T1L invasion and replication (24, 48) and on the viral and cellular components that mediate adherence of type 1 reoviruses to epithelial cell surfaces (22).

We have recently shown that the binding of type 1 reoviruses to rabbit M cells is mediated by the viral $\sigma 1$ protein (22). The ~50-kDa $\sigma 1$ protein is the hemagglutinin and attachment protein that also mediates binding of reoviruses to nonepithelial cells, including cultured fibroblasts and neurons (reviewed in references 31, 48, and 50). It is the serotype-determining protein, against which a neutralizing antibody response is most strongly directed (57). The $\sigma 1$ protein is sometimes visible by electron microscopy as long, 40- to 50-nm, bowed or bent fibers that extend from the fivefold axes of viral particles (19). $\sigma 1$ may also be capable of adopting a more contracted conformation in virions (16, 19). The $\sigma 1$ fiber is a homotrimer (13, 49) and is

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topped by a globular head domain formed by C-terminal sequences primarily in β -sheets (13, 18, 36). The fibrous tail of $\sigma 1$ is thought to comprise both α -helical coiled-coil and β -spiral domains (5, 13, 18, 19, 36). The globular head domain of $\sigma 1$ in both type 1 and type 3 reoviruses is required for efficient binding to fibroblasts and other cell types in culture (4, 10, 11), but the $\sigma 1$ region(s) involved in binding to M cell apical surfaces is not specifically known. One binding mechanism that appears common to many reovirus strains involves interaction of the $\sigma 1$ head domain with the recently identified protein receptor, junctional adhesion molecule 1 (JAM1) (4, 41). JAM1 is a cell surface glycoprotein that is present on nonpolarized cells and on the basolateral cell surfaces of intact epithelial layers (32). However, since it is not normally expressed on the apical surfaces of epithelial cells (32), JAM1 seems unlikely to mediate initial binding of reovirus to M cells in the intestinal epithelium.

Our recent report on reovirus binding to intestinal epithelial cells showed that specific glycoconjugates containing $\alpha 2,3$ -linked sialic acid on the apical surfaces of rabbit M cells and polarized Caco-2_{BBE} cells in culture are important for binding of type 1 reoviruses to these cells (22). In contrast, type 3 reoviruses fail to bind rabbit M cells and do not require $\alpha 2,3$ -linked sialic acid epitopes for binding to polarized Caco-2_{BBE} cells (22). This difference suggests that the interaction of $\sigma 1$ with particular sialic acid-containing glycoconjugates on epithelial cell apical surfaces is serotype specific and is consistent with previous evidence that the $\sigma 1$ proteins of type 1 and type 3 reoviruses have distinct carbohydrate-binding regions. In the $\sigma 1$ of reovirus T1L, a region required for hemagglutination, which is thought to involve carbohydrate-receptor binding, is located in the T(iv) distal tail region (8, 11). The presence of the head domain appears to be required for this activity of T1L $\sigma 1$, but the type 3 Dearing (T3D) $\sigma 1$ head can effectively substitute (11). In the $\sigma 1$ of T3D, a region required for binding to α -linked sialic acid (39) is located in the T(iii) middle tail region (8, 11, 12, 15, 35, 42), and the head domain is not required for this activity (11, 12, 35).

We have also recently characterized a new murine IgA monoclonal antibody (MAb), 1E1, which was raised against reovirus T1L and binds to T1L $\sigma 1$ (24). 1E1 is the first IgA MAb specific for the $\sigma 1$ protein. Its hybridoma clone was obtained by fusion of Peyer's patch cells after oral inoculation of mice with T1L, and it produces a mixture of IgA monomers, dimers, and higher polymers. When either passively administered or secreted into the intestine from subcutaneous hybridoma tumors, 1E1 protects mice from intestinal entry and mucosal replication of T1L after peroral challenge (24). The fact that mice with 1E1 in their intestinal secretions have no detectable virus in Peyer's patches after peroral challenge with T1L implies that this anti- $\sigma 1$ IgA prevents infection at an early stage, most likely by blocking entry to the mucosa through M cells. 1E1 neutralizes T1L infection of cultured murine fibroblasts (L929 cells) in vitro, blocks viral adherence to apical membranes of polarized Caco-2 cells, and therefore likely protects mice from peroral challenge by blocking the $\sigma 1$ -mediated adherence of viral particles to intestinal M cells (24). The epitope recognized by 1E1 appears to be discontinuous (24), but the regions of $\sigma 1$ recognized by 1E1 are not known. Defining the binding site of this protective, mucosally derived IgA

is important for understanding potential mechanisms of IgA-mediated immune protection. For example, the secreted antibody might bind directly to the carbohydrate-binding region of $\sigma 1$ involved in M cell adherence. Alternatively, it might interfere with this binding region by steric hindrance or might disrupt $\sigma 1$ function in some other way.

The importance of the head domain of T1L $\sigma 1$ for host cell infection and immune protection has been demonstrated with IgG MAb 5C6, which was raised against reovirus T1L, binds to the $\sigma 1$ protein of type 1 isolates (9, 51, 54, 55), and recognizes the $\sigma 1$ head domain (11). The exact location of the 5C6 epitope on $\sigma 1$ is not known. 5C6 blocks T1L binding and uptake in several cultured non-epithelial cell systems (4, 53, 54). When introduced into the blood circulation of mice either by intravenous injection (51) or from subcutaneous hybridoma tumors (24), 5C6 mediates protection against the systemic spread of T1L but does not prevent its entry into and infection of Peyer's patches after peroral challenge. This lack of mucosal protection by IgG MAb 5C6 in vivo is likely attributable to the incapacity of circulating IgG to undergo receptor-mediated secretion into the intestinal lumen of mice and the resulting lack of IgG in intestinal secretions at the time of challenge with virus (21). Indeed, when 5C6 is administered perorally to mice along with T1L, it is capable of preventing Peyer's patch infection (44). Thus, IgG MAb 5C6 and IgA MAb 1E1 share certain protective capacities. However, their respective binding sites on $\sigma 1$ could be distinct because induction of serum IgG and secretory IgA occur in different immune-sampling environments, function in different compartments, and may protect by different specific mechanisms.

In this study, we sought to identify the epitope on $\sigma 1$ that is recognized by IgA MAb 1E1, to compare it to that recognized by IgG MAb 5C6, and to determine the relationships of these epitopes to the putative epithelial carbohydrate-binding region of type 1 $\sigma 1$. We first confirmed the neutralizing activity of 1E1 against representatives of the three established reovirus serotypes in L929 cells. We then selected and characterized 1E1-resistant and 5C6-resistant mutants obtained from reovirus T1L. The results show that 1E1, like 5C6, recognizes the globular head domain of T1L. Both 1E1 and 5C6 recognize overlapping epitopes that span adjacent $\sigma 1$ monomers in the trimeric $\sigma 1$ head, and other evidence suggests these epitopes are not directly involved in carbohydrate-receptor binding. IgA MAb 1E1 thus most likely protects in vivo by sterically hindering interaction of a $\sigma 1$ carbohydrate-binding site with glycoconjugate receptors on apical surfaces of M cells in the intestinal epithelium. The results expand our understanding of the protective mucosal and systemic immune response against this enteric viral pathogen.

MATERIALS AND METHODS

L929 cells, reoviruses, and antibodies. Murine L929 cells were a laboratory stock maintained in spinner cultures in Joklik modified minimal essential medium (Irvine) supplemented to contain 2% fetal bovine serum and 2% calf bovine serum (Invitrogen) in addition to 2 mM glutamine, 100 U of penicillin/ml, and 100 μ g of streptomycin/ml (Mediatech). The same medium was used in growing virus stocks in L929 monolayers. For plaque assays, 2 $\times$ 199 medium (Irvine) supplemented to contain 4 mM glutamine (Mediatech) plus 200 U of penicillin/ml, 200 μ g of streptomycin/ml, and 0.5 μ g of amphotericin B (Fungizone; Invitrogen)/ml, with (standard assay) or without (protease overlay assay) 2% fetal bovine serum and 2% calf bovine serum (Invitrogen), was used.

Reoviruses T1L, T3D, 3HA1, 1HA3, T2/Human/Ohio/Jones/1955, and T3/

Murine/France/Clone 9/1961 were laboratory stocks derived from ones from the B. N. Fields laboratory. Reoviruses T1/Human/Netherlands/1/1984, T1/Human/Netherlands/1/1985, T2/Simian/Maryland/SV59/1958, T2/Human/Netherlands/1/1984, and T3/Human/Netherlands/1/1983 were laboratory stocks derived from ones from the T. S. Dermody laboratory (20). Purified virions were prepared with second-passage L929 cell lysates, and infectious subviral particles (ISVPs) were obtained after digestion with TLCK (*N* α -*p*-tosyl-L-lysine chloromethyl ketone)-treated α -chymotrypsin (Sigma) (16, 22).

IgA MAb 1E1 was obtained as a clarified hybridoma culture supernatant, diluted to an approximate concentration of 2 mg of MAb/ml, and stored frozen at -20°C since preparation (24). The 1E1 hybridoma supernatant contained a mixture of IgA monomers, dimers, and higher polymers as previously described (58). Purified stocks of IgG MAb 5C6 were obtained as a gift from K. L. Tyler, H. W. Virgin IV, and M. A. Mann. These stocks were obtained in phosphate-buffered saline (PBS) at an approximate concentration of 1 mg/ml and stored frozen at -80°C after purification (55).

Plaque reduction assays. Virus in a second- or third-passage cell lysate stock was diluted in PBS supplemented with 2 mM MgCl_2 and 0.4% (wt/vol) bovine serum albumin (Sigma) (PBS⁺/BSA) to an infectious concentration of 150 or 200 PFU per 100 μl . Undiluted MAb or PBS⁺/BSA (no-MAB control) was then added to the diluted virus stock as 1/20 of the final volume. The mixtures were incubated for 1 h at room temperature or 37°C (consistent for all samples within each experiment). The medium was removed from L929 monolayers in the wells of a six-well cluster plate, each monolayer was inoculated with 100 μl of the virus-MAB or control mixture, and virus-cell adsorption was allowed to proceed for 1 h at room temperature. For each mixture, three wells were inoculated within each experiment. After adsorption, the wells were overlaid with a 1:1 (vol/vol) mixture of completed 2×199 medium and 2% agar (Difco) and then analyzed by standard plaque assay (19). Plaques were counted after staining with neutral red (Sigma). The mean value from the three wells of each mixture was used in calculating the relative plaque number in the matched MAB and no-MAB samples. The results from one to four such experiments were then combined to give the results shown. In some other experiments not shown, different fivefold serial dilutions of the MABs were tested for their neutralization capacities against different viruses. In those experiments, MAB dilutions were made in PBS⁺/BSA and mixed in a 1:1 (vol/vol) ratio with a virus dilution containing 300 or 400 PFU per 100 μl .

Selections for neutralization-resistant mutants. A second- or third-passage cell lysate stock of T1L was mixed with MAB (1E1 or 5C6) at a 10:1 (vol/vol) ratio, followed by incubation at room temperature for 1 h. The medium was removed from an L929 cell monolayer in a 25-cm^2 flask, the monolayer was inoculated with the virus-MAB mixture, and viral adsorption was allowed to proceed for 1 h. At the end of this period, 5 ml of medium was added to the flask, and the flask was placed in a 37°C CO_2 incubator to allow viral growth. When 80 to 100% of the cells had detached from the flask, viral particles were released from the cells by freezing them at -80°C and then thawing them. This cell lysate was then used for the next passage. The different passage lysates in each series were subjected to protease-overlay plaque assay (as described in reference 25 except that α -chymotrypsin [Sigma] was used in place of trypsin) to determine their infectious titers before they were tested in a plaque reduction neutralization assay. Viral clones (plaques) that resisted neutralization in this assay were picked for further isolation and characterization. All clones characterized here were obtained from the first-passage lysate of each series. Each chosen plaque (putative 1E1- or 5C6-resistant mutant) was subjected to two more rounds of plaque isolation in protease overlay assays to promote clonal purity. The final plaque isolates were then subjected to serial passage in L929 cell monolayers to generate second-passage cell lysate stocks. The infectious titers of these stocks were again determined by protease overlay plaque assay before use in further experiments.

Enzyme-linked immunosorbent assay (ELISA) for MAB binding to virions. Maxi-Sorp 96-well plates (Nunc) were coated with wild-type T1L or neutralization-resistant mutants at 5×10^9 purified virions per well in virion buffer (150 mM NaCl, 10 mM MgCl_2 , 10 mM Tris [pH 7.5]) at 4°C overnight. After a blocking step with 3% (wt/vol) BSA (Sigma) in PBS, the plates were incubated with either MAB 1E1 at a starting concentration of 100 mg/ml or MAB 5C6 at a starting concentration of 50 mg/ml in serial threefold dilutions for 2 h at room temperature. The dilutions were performed in PBS containing 0.3% (wt/vol) BSA and 0.05% (vol/vol) Tween 20 (Sigma). After several washes with the dilution buffer, the plates were incubated with biotinylated goat anti-mouse anti-IgA or -IgG antibodies, respectively (Southern BioTech). The plates were then washed, and the bound MAB was detected by using streptavidin-horseradish peroxidase and tetramethyl benzidine Microwell substrate (Kirkegaard and Perry). Absorbances were read at 650 nm.

S1 nucleotide sequence determinations. Genomic reovirus RNA was isolated from purified virions by using TRIzol-LS reagent (Invitrogen). Briefly, 2×10^{12} virions were added to TRIzol-LS, mixed by pipetting, and incubated at room temperature for 5 min. Samples were then extracted with chloroform and incubated at room temperature for an additional 15 min. After centrifugation at $12,000\times g$, the aqueous phase was removed and mixed 1:2 (vol/vol) with isopropanol, and the precipitated RNA was pelleted by centrifugation at $12,000\times g$. RNA precipitates were washed with 75% ethanol, pelleted again by centrifugation at $7,500\times g$, allowed to air dry, and resuspended in diethyl pyrocarbonate-treated water. The genomic RNA was converted to DNA and amplified by using S1 forward primer 5'-GCTATTTCGCGCCTATGGATGC-3' and S1 reverse primer 5'-GATGATTGACCCCTTGTGCCGAGGGTTCG-3' (Invitrogen). Reverse transcription (RT) was done in two steps. First, 1 μl (1 μg) of genomic RNA, 1 μl (0.1 μg) of each primer, and 7 μl of diethyl pyrocarbonate-treated water were heated to 95°C for 5 min and then snap-cooled on ice. A mixture containing 1 μl of 10 mM deoxynucleoside triphosphates, 2 μl of $10\times$ reverse transcription (RT) buffer (Invitrogen), 4 μl of 25 mM MgCl_2 , 2 μl of 0.1 mM dithiothreitol, and 1 μl of RNaseOUT (Invitrogen) was next added to the primer-template mixture on ice. The new mixture was incubated at 42°C for 2 min before 1 μl of reverse transcriptase was added. The RT reaction was allowed to proceed at 42°C for an additional 48 min and then shifted to 70°C to terminate the reaction. Amplification of the RT products was carried out by mixing 5 μl of each reaction, 1 μl (0.1 μg) of each primer, 5 μl of $10\times$ Thermopol buffer (New England Biolabs), 1 μl of 10 mM deoxynucleoside triphosphates, 36 μl of water, and 1 μl of Vent polymerase (New England Biolabs) and then subjecting the mixture to the following steps: 95°C for 5 min; 36 cycles of 95°C for 45 s, 53°C for 45 s, and 70°C for 90 s; and finally 70°C for 10 min. The final DNA products were gel purified before sequencing, which was performed at the Dana-Farber/Harvard Cancer Center High-Throughput DNA Sequencing Facility according to their standard protocols (<http://dnaseq.med.harvard.edu/default.html>). Sequences were determined from both strands in full, except for primer-proximal sequences at each 5' end.

Molecular graphics. The crystal structure of the T3D σ 1 C-terminal half was displayed with RasMac 2.7.1 with coordinates obtained from The Protein Data Bank (entry 1KKE). The figure was assembled by using Photoshop 5.5 and Illustrator 8.0 (Adobe Systems).

Cell-binding studies. Experiments to characterize virus binding to M cells in tissue sections of rabbit Peyer's patches or to Caco-2_{BBE} cells in confluent monolayers were performed as previously described (22). Briefly, to detect virus binding to cells, purified ISVPs were biotinylated with EZ-Link Sulfo-NHS-Biotin (Pierce). Deparaffinized sections of fixed Peyer's patch tissue were overlaid with 200 μl of biotinylated ISVPs (3×10^{11} to 4×10^{11} particles/ml) in PBS containing 0.2% gelatin (Sigma) and incubated in a humidified chamber. Caco-2_{BBE} cell monolayers grown on glass coverslips were washed and incubated with virus by inversion of coverslips onto $50\text{-}\mu\text{l}$ droplets of biotinylated ISVPs (3×10^{11} to 4×10^{11} particles/ml) in gel saline solution (137 mM NaCl, 270 μM CaCl_2 , 840 μM MgCl_2 , 19 mM H_3BO_3 , 130 μM $\text{Na}_2\text{B}_4\text{O}_7$, 0.3% gelatin [pH 7.4]). Sections or coverslips were then washed and incubated with streptavidin-TRITC (tetramethyl rhodamine isothiocyanate) to label viral particles.

RESULTS

Serotype-restricted neutralization of type 1 reoviruses by IgA MAB 1E1. The plaque reduction assay in cell culture is the classical method for demonstrating antibody-mediated neutralization of viral particles (57). We used this assay to test the neutralizing capacity of 1E1 with several additional reovirus isolates (20, 23) than previously tested (24) in L929 cells. The results demonstrated that 1E1 neutralizes the two newly tested type 1 isolates, but neither of the two newly tested type 2 isolates nor the two newly tested type 3 isolates (Fig. 1). We conclude that 1E1 is capable of mediating serotype-specific neutralization of type 1 reoviruses, as recently shown to be determined by the σ 1-encoding S1 genome segment in the case of T1L (24). This is consistent with previous evidence that σ 1 is the serotype-determining protein of reoviruses (57), that 1E1 binds to σ 1 (24), and that 1E1 blocks binding of reovirus T1L particles to L929 cells (24).

FIG. 1. Serotype-specific neutralization of type 1 reoviruses by IgA MAb 1E1. Two additional field isolates representing each of the three established reovirus serotypes (six new isolates total) were evaluated for neutralization by 100 μ g of 1E1/ml in parallel with matched samples containing no MAb. The number of plaques in each 1E1-treated sample was expressed as a percentage of that in the matched no-MAb sample. Each bar represents the mean of three or four determinations $\pm$ the standard error. The newly tested isolates were T1/Human/Netherlands/1/1984 (T1N84), T1/Human/Netherlands/1/1985 (T1N85), T2/Human/Ohio/Jones/1955 (T2J), T2/Human/Netherlands/1/1984 (T2N84), T3/Murine/France/Clone 9/1961 (T3C9), and T3/Human/Netherlands/1/1983 (T3N83) (20, 23). The results for reoviruses T1L, T2/simian/Maryland/SV59/1958 (T2S59), and T3D, evaluated for neutralization by 1E1 in an identical manner, were previously reported (24) and were added to this figure for comparison. Representatives of a putative fourth serotype (2) were not tested.

Selection of T1L mutants that resist neutralization by 1E1.

Passage of reovirus T1L in the presence of 1E1 rapidly selected for a population of viruses resistant to neutralization by this MAb (Fig. 2A). Clones were isolated from the first-passage stock (i) because a large increase in neutralization resistance was already evident, (ii) to increase the chance of isolating clones with distinct mutations before the fittest mutants overwhelmed the stocks, and (iii) to increase the chance of isolating clones with single mutations. Seven clones were isolated by plaque purification from a 1E1-neutralized sample of the stock (Fig. 2A), serially plaque purified to promote clonal purity, and subjected to serial passage to generate high-titer stocks. Plaque reduction assays with these new stocks revealed that three of the clones are indeed 1E1-resistant mutants (Fig. 2B and Table 1). Six additional 1E1-resistant mutants were identified among seven clones selected from a second, clonally distinct stock of reovirus T1L by using the same protocol (Fig. 2D and E and Table 1). This second stock of T1L was used to guarantee the selection of mutants with independent origins which possibly contained distinct mutations that can interfere with 1E1-mediated neutralization.

Selection of T1L mutants that resist neutralization by IgG MAb 5C6. Seven of the nine 1E1-resistant mutants also showed resistance to neutralization by 5C6 (51, 54, 55) in initial screens (Fig. 2C and F and Table 1). This finding suggested that 1E1 and 5C6 bind to overlapping regions of σ 1 (see below). In an effort to identify additional mutations that affect binding to 5C6, we isolated seven putative 5C6-resistant mutants of reovirus T1L by using the same protocol as that used to generate the 1E1-resistant mutants above, except that 5C6 was added during passage (Fig. 3A). Plaque reduction assays revealed

that six of these clones are indeed resistant to 5C6 neutralization (Fig. 3B and Table 1). All six of these 5C6-resistant mutants also showed resistance to 1E1 (Fig. 3C and Table 1).

MAb-binding phenotypes of the mutants. Neutralization-resistant mutants almost always have mutations within the epitope to which the neutralizing MAb binds, and the mutations thus act directly to reduce MAb binding rather than through some indirect mechanism (7, 43, 45). Indirect mechanisms are nonetheless conceivable (45). We used an ELISA to determine whether the 1E1- and 5C6-selected mutants showed reduced binding to either 1E1 or 5C6. Five of the mutants selected for 1E1 resistance and two of the mutants selected for 5C6 resistance were tested. All seven of the mutants showed reduced binding of both MAbs (Fig. 4 and data not shown). As for relative effects on 1E1 and 5C6 binding, mutants could be grouped into two categories: one for which 1E1 binding was more strongly affected than 5C6 binding (L1E1-a2 and L1E1-a7) and another for which the opposite was true (L1E1-b5, L1E1-b6, L1E1-b7, L5C6-a3, and L5C6-a5) (Fig. 4, Table 1, and data not shown). These binding results correlated with those from neutralization experiments (Fig. 2, 3, and 4) and provided further evidence that 1E1 and 5C6 bind to overlapping regions of σ 1.

Sequence substitutions in the S1 genome segments and encoded σ 1 proteins of the 1E1- and 5C6-resistant mutants. To localize the probable regions to which 1E1 and 5C6 bind in the σ 1 protein, we determined the nucleotide sequences of the S1 genome segments of the two parental T1L clones, the seven mutants characterized by ELISA, and five of the eight other mutants (Table 1). Sequences were directly determined by cycle sequencing after amplification of DNA copies of the respective S1 genomic RNA segment. Relative to the S1 sequence of its respective parent, each mutant contained either one or two nucleotide changes that caused either one or two amino acid changes in the deduced σ 1 protein sequence (Table 1). Each mutant selected for 1E1 resistance showed a change at one of three amino acid positions (415, 417, and 445), and each mutant selected for 5C6 resistance showed changes at one or two of three amino acid positions as well (417, 447, and 251 and 417) (Table 1). A mutation at one of these sites (Gln417Lys) was shared between the two sets of mutants, providing further evidence that 1E1 and 5C6 bind to overlapping epitopes. All but one of the mutations was found in the region between amino acids 415 and 447, which constitutes part of the head domain of T1L σ 1 (13, 18, 19, 36). The one mutation outside this region, Ile251Thr, was found in a mutant (L5C6-a3) that also contained a Gln417Lys mutation, and we consider it likely that the Ile251Thr mutation arose by chance in this clone and does not contribute to its MAb resistance phenotypes (Fig. 3 and 4; see also below). The two parental T1L sequences were identical but differed from the published T1L sequences (17, 36) at two nucleotide positions (C393A and A1416G), which caused two changes in the deduced σ 1 protein sequence (Ser127Tyr and Asn468Ser) and one change in the deduced σ 1s protein sequence (Pro107Thr). All 12 of the 1E1- and 5C6-resistant mutants for which S1 sequences were determined contained these two additional nucleotide changes relative to the published T1L S1 sequences and were thus identical to their parents in that regard.

FIG. 2. Generation and analysis of 1E1-resistant mutants. Virus stocks were evaluated for neutralization by 100 μ g of 1E1/ml or 50 μ g of 5C6/ml in parallel with matched samples containing no MAb. The number of plaques in each 1E1- or 5C6-treated sample was expressed as a percentage of that in the matched no-MAB sample. (A and D) Two clonally independent stocks of reovirus T1L [designated (a) and (b)] were subjected to serial passage in the presence of IgA MAb 1E1, and the resulting passages were evaluated for neutralization by 1E1. Each bar represents the value of a single determination. Each original T1L stock is designated passage 0. For each passage series, the arrows indicate when 1E1 was added, and the asterisk indicates the sample from which seven putative 1E1-resistant mutants were isolated by plaque purification. (B, C, E, and F) The total of 14 putative 1E1-resistant mutants described in panels A and D were evaluated for neutralization by 1E1 (B and E) or 5C6 (C and F) in parallel with the original T1L stock from which each mutant was, respectively, derived. The stacked bars represent the results of two independent determinations. The names of the clones examined for MAb binding in Fig. 4 are boxed.

Locations of the 1E1- and 5C6-selected mutations in the $\sigma 1$ structure. The structure of the C-terminal half of the T3D $\sigma 1$ trimer (amino acids 246 to 455) was recently determined at 2.6 Å by X-ray crystallography (13). Alignment of the T1L and T3D $\sigma 1$ sequences (17, 36) allowed conserved regions to be identified and then located in the T3D structure (13). The conserved regions include a surface loop in the head domain that is proposed to interact with the cell surface protein receptor JAM1 (13, 41) (Fig. 5). Although the T1L $\sigma 1$ structure is not yet known, the T3D $\sigma 1$ trimer structure can be used as a model to locate the approximate locations of the changes that impart 1E1 and 5C6 resistance to the current set of T1L mutants. Amino acids 246 to 455 in the T3D $\sigma 1$ crystal structure correspond to amino acids 258 to 470 in the aligned T1L $\sigma 1$ sequence (17, 36). The resistance-associated changes at amino acids 415, 417, 445, and 447 in T1L $\sigma 1$, respectively, align with amino acids 400, 402, 430, and 432 in T3D $\sigma 1$ (17, 36). All four of these residues are partly exposed on the side surface of the

T3D $\sigma 1$ trimer head and cluster within 25 Å of each other across an interface between two adjacent subunits (Fig. 5). There are three such clusters per trimer. Within each of these putative MAb-binding regions, residues 415 and 417 are contributed by one subunit and residues 445 and 447 by the adjacent subunit (Fig. 5). Since mutations on both sides of each interface reduce binding of both 1E1 and 5C6 (Fig. 4), we conclude that these MAbs bind to substantially overlapping regions that span the subunit interface. These findings suggest that binding of 1E1 and 5C6 is likely dependent on the intact quaternary structure of the trimeric $\sigma 1$ head domain, a finding consistent with previous evidence that both 1E1 and 5C6 recognize a complex epitope that is sensitive to $\sigma 1$ folding (24, 55). Amino acid 251 in T1L $\sigma 1$, which was identified as the site of a second mutation in 5C6-resistant mutant L5C6-a3 (Table 1), is outside the region visualized in the T3D $\sigma 1$ crystal structure (13) and is thus distant from the other neutralization resistance mutations in the protein structure. This finding sup-

TABLE 1. Properties of 1E1- and 5C6-resistant mutants for which S1 sequences were determined

Virus strain ^a	Resistance to neutralization by 1E1/5C6 ^b	Resistance to binding by 1E1/5C6 ^b	Mutation(s) in S1 genome segment ^c	Mutation(s) in σ 1 protein ^d	P/PFU ratio ^e
T1L(a)	-/-	-/-	NA	NA	82
T1L(b)	-/-	-/-	NA	NA	125
L1E1-a1	++/-	NT/NT	A1346C	N445H	NT
L1E1-a2	++/-	++/+	G1256U	A415S	145
L1E1-a7	++/+	++/+	C1262G	Q417E	107
L1E1-b5	++/++	++/+	C1262A	Q417K	96
L1E1-b6	++/++	++/+	C1262A	Q417K	96
L1E1-b7	++/++	++/+	C1262A	Q417K	95
L5C6-a2	++/++	NT/NT	C1262A	Q417K	NT
L5C6-a3	++/++	++/+	U752C, C1262A	I251T, Q417K	80
L5C6-a4	++/++	NT/NT	G1352A	G447S	NT
L5C6-a5	++/++	++/+	G1352A	G447S	111
L5C6-a6	++/++	NT/NT	C1262A	Q417K	NT
L5C6-a7	++/++	NT/NT	C1262A	Q417K	NT

^a Two independent clones of reovirus T1L from which mutants were selected are indicated by "(a)" and "(b)". These designations are also found in the names of the mutants to indicate the parental clone of origin. The MAb used to select each mutant is also found in its name.

^b Assays are described in Materials and Methods. The behavior of each T1L parent is indicated as minus (-), and the behavior of each mutant is indicated by the relative degree of change from that of T1L (+ or ++). NT, not tested.

^c S1 nucleotide sequence determinations were performed as described in Materials and Methods. Changes are indicated by the plus-strand position number flanked by the wild-type nucleotide on the left and the mutant nucleotide on the right. NA, not applicable.

^d σ 1 amino acid sequences were deduced from the S1 nucleotide sequences. Changes are indicated by the position number flanked by the wild-type amino acid on the left and the mutant amino acid on the right. Amino acids are indicated by single-letter code. NA, not applicable.

^e The particle/PFU ratio (P/PFU) is an indication of relative infectivity of purified virions of each clone. NT, not tested.

ports the conclusion that the mutation at residue 251 does not contribute to the resistance phenotype of that mutant.

Cell adherence phenotypes of the mutants. In an effort to link other defects or changes with the neutralization resistance mutations in T1L σ 1 identified in the present study, we analyzed the 1E1- and 5C6-resistant mutants with regard to several other properties. In hemagglutination assays, the mutants were indistinguishable from their wild-type parents (data not shown). They were also indistinguishable from the parents with regard to relative infectivity in L929 cells (Table 1), which is not surprising since these are the cells in which the mutants were selected and amplified. ISVPs of representative mutants were next compared to those of the T1L parents for attachment to M cells in an overlay assay on tissue sections of rabbit Peyer's patches (22). Each mutant mimicked its parent in that each showed selective adherence to M cells in the sections (Fig. 6, Table 2, and data not shown). In addition, each mutant mimicked its parent with regard to inhibition of this adherence when the sections were preincubated with the lectins MAL-I and MAL-II, which specifically recognize oligosaccharides containing α 2,3-linked sialic acid, but not when the sections were preincubated with the lectin SNA, which specifically recognizes oligosaccharides containing α 2,6-linked sialic acid (Fig. 6, Table 2, and data not shown) (22). As a final test, ISVPs of the same representative mutants were compared to those of their parents for attachment to confluent, polarized Caco-2_{BBE} cell monolayers (22), and no differences were observed between mutants and parents with regard either to adherence to these cells or to the pattern of adherence inhibition by the preceding lectins (Table 2 and data not shown). Thus, the defined alterations in the σ 1 proteins of the 1E1- and 5C6-resistant mutants did not affect their capacity to recognize specific glycoconjugates containing α 2,3-linked sialic acid and to adhere to epithelial cell apical surfaces.

DISCUSSION

During its pathogenesis in the intestine of adult mice, reovirus T1L first interacts with the apical surfaces of epithelial M cells to enter transcytotic vesicles and cross the epithelial barrier; once in the mucosa, the virus then binds to target cells, such as the basolateral membranes of epithelial cells, to initiate a productive infection (60, 61; reviewed in references 34 and 37). Our recent work has suggested that IgA MAb 1E1, directed against the reovirus T1L σ 1 protein, can protect the intestine potentially at both stages, by blocking attachment to epithelial cell apical membranes and thereby preventing entry into the mucosa and also by blocking infection of target cells, as shown by neutralization of infectivity in L929 cells (24). The results of the present study show that this protective and neutralizing IgA antibody and a protective and neutralizing IgG antibody, 5C6, recognize closely related epitopes that span adjacent σ 1 monomers in the trimeric σ 1 head domain.

Binding of type 1 reoviruses to apical membranes of epithelial cells, where the receptor protein JAM1 is not normally present (32), involves particular carbohydrate structures (22). We have recently shown that glycoconjugates containing α 2,3-linked sialic acid on apical M cell surfaces are important for the σ 1-based binding of type 1 reoviruses to those cells (22). The part(s) of σ 1 required for this binding activity remain to be determined but could involve the distal region of the type 1 σ 1 tail [T(iv)] that is required for hemagglutination (11) or another, as-yet-unknown carbohydrate recognition domain in this protein. The 1E1 and 5C6 results reported here are consistent with the hypothesis that the head domain of type 1 σ 1 is important for binding to M cells in vivo. The 1E1 and 5C6 epitopes in the σ 1 head may not appear to be well situated for steric hindrance of the carbohydrate-binding region in the σ 1 tail, but it is important to note that antibody molecules are

FIG. 3. Generation and analysis of 5C6-resistant mutants. Viral stocks were evaluated for neutralization by 1E1 or 5C6 as described in Fig. 2. (A) A stock of reovirus T1L [same stock (a) as in Fig. 2] was subjected to serial passage in the presence of IgA MAb 5C6 (see Materials and Methods), and the resulting passages were evaluated for neutralization by 5C6. Each bar represents the value of a single determination. The original T1L stock is designated passage 0. The arrow indicates when 5C6 was added in the passage series, and the asterisk indicates the sample from which seven putative 5C6-resistant mutants were isolated by plaque purification. (B and C) The seven putative 5C6-resistant mutants described in panel A were evaluated for neutralization by 5C6 (B) or 1E1 (C) in parallel with the original T1L stock from which they were derived. The stacked bars represent the results of two determinations. The names of the clones examined for MAb binding in Fig. 4 are boxed.

relatively large. Indeed, IgA dimers visualized by electron microscopy are 30 to 35 nm in length (14), and therefore antibody binding to the 1E1 or 5C6 epitope could theoretically block interaction of both the head and tail domains of $\sigma 1$ with cell surface receptors. Moreover, the carbohydrate structures that contribute to the receptors for type 1 reoviruses on intestinal epithelial cells may not be well exposed on the luminal cell surface but rather may be located on the oligosaccharide side chains of large, complex membrane glycoproteins (29, 30) or on glycolipids (26) sequestered under the membrane glycoprotein coats of intestinal epithelial cells. This idea is supported by the observation that the lectin MAL-II, which blocks binding of type 1 reoviruses to epithelial cells (22), binds to rabbit M cells when it is applied to the mucosa in soluble form but not when it is conjugated to 1- μ m microparticles (33). We have

previously observed that proteolytic conversion of T1L virions to ISVPs is required for M-cell binding in mice (1) and have hypothesized that extension of the $\sigma 1$ trimer from the surface of ISVPs may promote M cell binding by allowing the head region to penetrate the apical surface glycoprotein coats of

FIG. 4. Binding of 1E1 or 5C6 to the $\sigma 1$ protein in purified virions of T1L or T1L-derived MAb-resistant mutants. A microplate ELISA was performed by using different concentrations of the MAbs 1E1 and 5C6, as indicated, to bind to plate-bound virions. Values are expressed as optical density (OD) units from the detection system described in Materials and Methods. Each symbol represents the mean of three or four determinations $\pm$ the standard error. In each panel, values for MAb 1E1 are shown as open symbols connected by solid lines, whereas values for MAb 5C6 are shown as symbols with cross-hairs connected by dashed lines. The symbols represent T1L(a) (squares), L1E1-a2 (diamonds), and L1E1-a7 (circles) in panel A and T1L(b) (squares), L1E1-b5 (diamonds), and L1E1-b6 (circles) in panel B. The results for L1E1-b7 were also obtained in these experiments and were indistinguishable from those shown for L1E1-b5 and L1E1-b6. The symbols represent T1L(a) (squares), L5C6-a3 (diamonds), and L5C6-a5 (circles) in panel C. These samples were analyzed in the same experiments as for panel A, and so the data for T1L(a) are the same as presented in panel A.

FIG. 5. Approximate locations of the 1E1 and 5C6 resistance mutations based on the T3D $\sigma 1$ crystal structure. The crystal structure of the reovirus T3D $\sigma 1$ trimer (13) is shown in wall-eyed stereo view and space-filling format, with each subunit colored a different shade of blue. The positions of $\sigma 1$ mutations in the A2- and G5-resistant mutants of T3D (6, 46) are shown in yellow in each subunit. The positions of $\sigma 1$ mutations in the 1E1- and 5C6-resistant mutants of reovirus T1L (the present study) are shown in red in each subunit. The pattern and colors of the mutant positions are replicated in the space between the two trimer images and labeled with the position numbers of the mutations. For the 1E1- and 5C6-resistant mutants, each mutation is indicated by position in the T1L $\sigma 1$ sequence (upper), as well as by position in the aligned T3D $\sigma 1$ sequence (lower) (17, 36). The latter positions are the ones mapped on the T3D $\sigma 1$ trimer structure. A red oval indicates the approximate footprint (13 to 30 amino acids [7, 40]) expected to be covered or contacted by one epitope-binding Fab region centered around the identified mutations. Two other subunit interfaces flanked by MAb resistance mutations are hidden on the back of the trimer in this view. Residues 429 and 442 are shown in stick format to reveal residues 430 (T1L 445) and 402 (T1L 417) more fully. The putative JAM1 binding loop (4, 13) is shown in dark green in each subunit. The region of T1L $\sigma 1$ that contains type-specific determinants of hemagglutination (i.e., carbohydrate receptor binding) (11) is shown in light green for the subunit otherwise shown in light blue.

epithelial cells and reach sequestered binding sites near the plasma membrane (33). Attachment of IgA antibodies to the $\sigma 1$ head might therefore not only sterically hinder receptor binding but also impede access of the extended $\sigma 1$ trimer to sequestered receptor sites on epithelial apical surfaces.

To infect target cells within the mucosa, type 1 reoviruses may exploit both carbohydrate structures and membrane proteins as receptors. Through analysis of antibody escape mutants in L929 cell infectivity assays, we have shown that the epitopes recognized by IgG MAb 5C6 and IgA MAb 1E1 overlap. Both antibodies block binding of T1L to fixed L929 cells and neutralize T1L infectivity, and although their affinities for $\sigma 1$ may differ, both antibodies most likely protect target cells by the same mechanism. Neutralizing antibodies often act at the surfaces of target cells, by hindering virus-receptor interactions (28, 45, 59). A conserved loop in the $\sigma 1$ head is proposed to be an important part of the JAM1 binding site on target cells including L929 cells (13). 1E1 and 5C6 binding near

the top of the $\sigma 1$ head, as revealed by the present results, appear well situated to effect steric hindrance of JAM1 binding at the proposed sites (13), especially if the contact regions are larger than currently suggested and extend laterally toward the $\sigma 1$ subunit interfaces (13). However, stable attachment of reoviruses leading to endocytic uptake from cell surfaces may be a multistep process involving successive or concurrent interactions with lower- and higher-affinity carbohydrate and protein receptor molecules (3), and a particular neutralizing antibody may interfere with one or more of these steps. We have suggested that type 1 reoviruses may bind to specific glycoconjugates containing $\alpha 2,3$ -linked sialic acid on M-cell surfaces (22), and these carbohydrate structures may be present on target cells as well. Both 5C6 (44, 55) and 1E1 (24; data not shown) are effective at blocking agglutination of human erythrocytes and attachment to M cells, suggesting they do indeed interfere with carbohydrate-receptor binding by some mechanism. However, binding of type 1 reoviruses to L929 cells is clearly not

FIG. 6. Binding to rabbit M cells by reovirus 1E1-resistant mutant L1E1-a7. Deparaffinized 5- μ m sections of rabbit Peyer's patch were incubated with or without MAL-II lectin, washed, and overlaid with biotinylated ISVPs, which were then visualized with streptavidin-TRITC. In all micrographs, a representative follicle-associated epithelium (FAE) and the epithelium of an adjacent villus are shown. (A) ISVPs of the parental T1L strain adhered to M cell apical surfaces (arrows) in the follicle-associated epithelium (FAE; arrowhead). (B) In the absence of MAL-II, L1E1-a7 ISVPs adhered to M-cell apical surfaces in a pattern indistinguishable from that of the parent strain. (C) M cells in adjacent sections of Peyer's patch mucosa preincubated with MAL-II, in contrast, did not bind L1E1-a7 ISVPs. Bar, 25 μ m.

dependent on the specific carbohydrate structures that contribute to the receptors for these viruses on epithelial cells, because L929 cell binding is not inhibited in the presence of MAL-II lectin (22). In any case, if carbohydrate recognition sites on type 1 $\sigma 1$ play a role in L929 cell infection, their capacity for such function is greatly reduced in the presence of 1E1 and 5C6. Taken together, the data suggest that these antibodies sterically hinder the binding of type 1 reoviruses to both JAM1 and carbohydrate receptors on target cells.

Neutralizing antibodies can hinder attachment by other mechanisms as well. For example, they can induce conformational changes in the viral surface proteins that reduce affinity for receptor binding at an adjacent or more distant site (28, 45). This mechanism seems plausible for the adjacent, pro-

posed JAM1-binding sites in T1L $\sigma 1$ but possibly less plausible for the putatively more distant carbohydrate-binding sites. Nonetheless, by binding across the subunit interfaces in the $\sigma 1$ head, 1E1 and 5C6 might contort the intratrimer interactions and the receptor-binding regions in turn, even at some distance from the MAb-binding sites. The discovery that the overlapping 1E1 and 5C6 epitopes span subunit interfaces in the $\sigma 1$ head suggests an additional, distinctive mechanism of neutralization, namely, that 1E1 and 5C6 may "staple" together the trimeric heads such that they cannot dissociate into their constituent monomers. Dissociation of the heads may be required, for example, to allow each trimer to mediate multivalent attachment in which two, or even all three, of the dissociated heads concurrently bind to separate protein or carbohydrate receptor molecules. Multivalent attachment to cell surface receptors may be required for reovirus infection (3), so that blocking it may be an effective means of neutralization. In considering this hypothesis, it is notable that mutants of reovirus T3D resistant to the neutralizing IgG MAb G5 (8, 46) have a change at $\sigma 1$ residue 340 or 419 (6), which also approach each other from opposite sides of a subunit interface in the trimeric T3D $\sigma 1$ head (13) (Fig. 5). Because residues 340 and 419 in T3D $\sigma 1$ are near the proposed JAM1-binding sites in the structure (13), it is reasonable to hypothesize that G5 mediates neutralization by sterically hindering JAM1 binding or by inducing conformational changes in nearby regions that reduce affinity for the receptor. Because the G5 epitope also spans the subunit interface, however, it may be that neutralization is enhanced or wholly mediated by G5 stapling together the trimeric head of T3D $\sigma 1$, as we suggest above for 1E1 and 5C6 on T1L $\sigma 1$. The capacity of the $\sigma 1$ heads to undergo monomer-trimer transitions during the course of cell entry has been previously predicted based on several features of the T3D $\sigma 1$ crystal structure (13, 48).

The mutations identified in the present study indicate that the two type 1 $\sigma 1$ -specific neutralizing MAbs 1E1 and 5C6 bind to essentially the same region of T1L $\sigma 1$. This was corroborated by the capacity of 5C6 to compete with 1E1 binding to viral particles in dot blot assays (data not shown). Not only were these two MAbs generated at different times and in different labs, but they were generated with cells from different lymphatic organs (Peyer's patch versus spleen) and are of different classes (IgA versus IgG), proving their independent

TABLE 2. Binding profiles of representative 1E1- and 5C6-resistant mutants^a

Virus mutant	Binding to Peyer's patch sections				Binding to Caco-2 _{BBE} monolayers			
	No lectin	MAL-I	MAL-II	SNA	No lectin	MAL-I	MAL-II	SNA
L1E1-a2	+	—	—	+	+	—	—	+
L1E1-a7	+	—	—	+	+	—	—	+
L1E1-b5	+	—	—	+	+	—	—	+
L1E1-b6	+	—	—	+	+	—	—	+
L1E1-b7	+	—	—	+	+	—	—	+
L5C6-a3	+	—	—	+	+	—	—	+
L5C6-a5	+	—	—	+	+	—	—	+

^a Binding of reovirus ISVPs was assayed as described in Helander et al. (22), either without preincubation of sections or cells with lectin (No lectin) or with preincubation of sections or cells with 10 μ g of the lectins MAL-I, MAL-II, or SNA/ml. +, Binding to M-cell apical surfaces; —, complete inhibition of binding.

origins. The discovery that they bind to the same region of $\sigma 1$ suggests that (i) antibodies of these two distinct classes are mediating neutralization by the same mechanism, (ii) antibody binding to that region is especially effective at neutralization, and/or (iii) this region is especially immunogenic. A mutation at residue 419 in T3D $\sigma 1$, which is present in most of the G5-resistant mutants, is also present in the two T3D mutants selected for resistance to another type 3 $\sigma 1$ -specific neutralizing IgG MAbs, A2 (6, 8). In addition, A2 and G5 compete for binding to viral particles in radioimmunoassays (47). A2 and G5 therefore appear to bind to very similar regions, which in turn are near the regions bound by 1E1 and 5C6 (Fig. 5). Intraperitoneally administered G5 is protective against reovirus type 3 infections of target cells outside the mouse intestine (51, 52, 56). Thus, four strongly neutralizing and protective IgA or IgG MAbs—A2, G5, 1E1, and 5C6—all bind to adjacent regions on the side of the $\sigma 1$ head and across the interface between $\sigma 1$ subunits. Because these epitopes all span subunit interfaces in the $\sigma 1$ head, they are likely to require the intact quaternary (trimer) structure of the head for binding. Additional $\sigma 1$ -specific neutralizing MAbs and the capacity to map their binding epitopes should contribute to further understanding the mechanisms of neutralization and protection in the reovirus system.

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