Protective Antibodies Inhibit Reovirus Internalization and Uncoating by Intracellular Proteases

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We identified in vitro correlates of in vivo protection mediated by nonneutralizing antibodies specific for reovirus capsid proteins. We defined mechanisms of antibody action by analyzing monoclonal antibody (MAb) effects at sequential steps in reovirus serotype 3 strain Dearing (T3D) infection of L cells. Two types of experiments showed that protective MAbs specific for the outer capsid proteins $\sigma 3$ or $\mu 1$ inhibited T3D infection independent of effects on binding. First, MAbs which had no effect on T3D binding inhibited T3D growth. Second, MAb-coated T3D attached to L cells did not replicate as efficiently as T3D without bound antibody. We therefore defined $\sigma 3$ -specific MAb effects on postbinding steps in T3D infection. T3D coated with MAb $\sigma 3$ -10G10 exhibited prolonged sensitivity to growth inhibition by ammonium chloride. Since ammonium chloride inhibits endosomal acidification and proteolytic processing of the T3D capsid, this suggested that MAbs inhibit early steps in T3D infection. This was confirmed by direct demonstration that several $\sigma 3$ -specific MAbs inhibited proteolytic uncoating of virions by fibroblasts. We identified two mechanisms for antibody-mediated inhibition of virion uncoating: (i) inhibition of internalization of T3D-MAb complexes bound to the cell surface, and (ii) inhibition of intracellular proteolysis of the T3D capsid. Studies using a cell-free system confirmed that $\sigma 3$ -specific MAbs directly block proteolytic uncoating of the T3D virion. In addition, we found that $\sigma 3$ -specific MAbs block (and therefore define) two distinct steps in proteolytic uncoating of the reovirion. We conclude that antibodies which are protective in vivo inhibit postbinding events in reovirus infection of permissive cells. Protective antibodies act by inhibiting internalization and intracellular proteolytic uncoating of the virion. Analysis of postbinding mechanisms of MAb action may identify targets for vaccine development and antiviral therapy.

Despite the importance of humoral immunity in protection against viral infection, the mechanisms by which antibodies alter viral pathogenesis in vivo and inhibit viral replication in vitro are poorly understood. We recently isolated monoclonal antibodies (MAbs) specific for the outer capsid proteins of reoviruses and characterized them in vivo and in vitro (25, 32, 35). The capacity of these MAbs to protect mice against reovirus infection is not explained by such in vitro properties as antibody isotype, binding avidity, plaque reduction neutralization, or hemagglutination inhibition. In order to identify potential in vitro correlates of antibody-mediated protection in vivo, we investigated the effects of protective MAbs at sequential stages in reovirus infection of permissive cells in culture.

Reovirus T3D binds to sialic acid residues on nucleated cells and erythrocytes through the $\sigma 1$ cell attachment protein (17, 23, 24, 38). Following attachment to the cell, virions are internalized by a process analogous to receptor-mediated endocytosis (reviewed in references 8 and 21). Internalization and intracellular transport of reovirus are associated with structural and conformational changes in the virion. These include: (i) proteolysis and removal of outer capsid protein $\sigma 3$ and (ii) cleavage of $\mu 1$ c to δ and ϕ (8, 20). These changes result in the formation of infectious subviral particles (ISVPs) (reviewed in references 8 and 21). These proteolytic events generate dramatic structural transitions in the viral capsid and are critical early steps in replication. Proteolytic uncoating of

virions by cells is mimicked by in vitro digestion of virions to ISVPs by tosyllysine chloromethyl ketone-treated alpha-chymotrypsin (chymotrypsin) (4, 16, 30). ISVPs remain infectious but are not transcriptionally active. Further proteolytic cleavage of outer capsid proteins of ISVPs removes δ and $\sigma 1$, generating noninfectious but transcriptionally active core particles (5, 26). Cellular proteolytic uncoating of virions to ISVPs is inhibited by the lysosomotropic agent ammonium chloride, suggesting that proteolytic uncoating of virions occurs in an acidic endosomal compartment (6, 19, 30). Growth of ISVPs generated in cell-free systems by chymotrypsin digestion is not inhibited by ammonium chloride. This shows that, once uncoating of virions to ISVPs has been accomplished, there is no further requirement for endosomal acidification for completion of subsequent steps in the replicative cycle (30).

Knowledge of these early steps in reovirus infection allowed us to analyze the effects of protective MAbs specific for reovirus outer capsid protein $\sigma 3$ on sequential early steps in viral replication. In this paper we demonstrate that protective $\sigma 3$ MAbs inhibit T3D growth and intracellular proteolytic uncoating of the virion independent of effects on binding. Postbinding mechanisms by which protective MAbs inhibit reovirus growth include inhibition of (i) internalization of virus and (ii) proteolytic uncoating of virions to ISVPs in cells. In vitro studies showed that one likely mechanism for MAb-mediated inhibition of intracellular proteolytic uncoating of the virion is direct inhibition of capsid protein proteolysis.

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TABLE 1. Designations, specificities, neutralizing capacity, and in vivo protective capacity of MABs

MAB	Specificity ^a	Neutralization ^b ($\mu\text{g/ml}$)	Plaque morphology	Protection ^c
σ 1T3D-G5	σ 1 (T3D)	<0.03	Normal	++
σ 1T1L-5C6	σ 1 (T1L)	>100	Normal	—
μ 1-10F6	μ 1	>100	Normal	—
μ 1-8H6	μ 1	$\geq$ 100	Small	++
σ 3-8F12	σ 3	>100	Normal	—
σ 3-4F2	σ 3	>100	Small	++
σ 3-10C1	σ 3	>100	Small	++
σ 3-10G10	σ 3	10–30	Small	++

^a See reference 35.^b Lowest concentration of MAB giving 80% reduction in plaque number (32).^c As indicated by survival of 1- to 2-day old NIH Swiss mice receiving 100 μg of MAB prior to challenge with 100 to 1000 $\times$ 50% lethal dose (LD_{50}) of T3D intracranially or intramuscularly (32). ++, significant protection; —, no protection.

MATERIALS AND METHODS

Viruses and MABs. T1L and T3D were obtained from laboratory stocks. Methods for virus growth, purification, quantitation, storage, and plaque assay were as published previously (32, 35). Radiolabelled T3D was prepared by adding [³⁵S]methionine (2.5 to 5.0 mCi/2.5 to 5 $\times$ 10⁸ L cells; ICN Biomedicals, Irvine, Calif.) for 72 h of viral growth in spinner culture followed by cesium chloride gradient purification of virions. ³⁵S-T3D was dialyzed against dialysis buffer (150 mM NaCl, 15 mM MgCl₂, 10 mM Tris [pH 7.4]) and quantitated by optical density (OD) at 260 nm (1 OD₂₆₀ unit = 2.1 $\times$ 10¹² virion particles = 185 μg of viral protein [27]). Protein A-purified MABs were prepared and stored as described previously (35). Designations, specificities, plaque reduction neutralization capacities, and in vivo protective capacities of MABs used in this study were defined in prior publications (32, 35) and are summarized in Table 1.

Tissue culture media. All media contained 100 U of penicillin per ml and 100 μg of streptomycin per ml (Biofluids, Rockville, Md., or Irvine Scientific, Santa Ana, Calif.). L cells were maintained in spinner and monolayer culture using minimal essential medium (MEM) containing 5% fetal calf serum (FCS) (HyClone, Ogden, Utah, or Biofluids) and 2 mM L-glutamine (Biofluids) (MEM⁺). Hanks balanced salt solution (HBSS) (Biofluids) containing 1 to 5% FCS and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Biofluids) (HBSS⁺) was used for MAB dilutions and room air incubations on ice. RPMI 1640 (Biofluids) supplemented with 10% fetal calf serum and 2 mM L-glutamine (RPMI⁺) or MEM⁺ was used for viral processing and internalization assays performed at 37°C in 5% CO₂.

Neutralization. Neutralization assays were carried out as previously described (32). Briefly, T3D was mixed with MABs at 0.03 to 100 $\mu\text{g/ml}$ for 1 h at room temperature and was then plated as for plaque assay. On day 7, plaques were counted and the number was compared with the number of plaques present in either wells with no antibody or wells with control MAB (σ 1T1L-5C6). An 80% reduction in the number of plaques was defined as significant neutralization (28, 37). We noted that several MABs did not reduce plaque number by >80% but did decrease the size of plaques at concentrations of 30 to 100 $\mu\text{g/ml}$. For this reason both 80% plaque reduction titers and the morphologies of plaques at high MAB concentrations are reported (Table 1).

Single-cycle growth of T3D. L cells (5 $\times$ 10⁵ per well) were plated in 24-well clusters (Costar, Cambridge, Mass.) and were

incubated overnight. Clusters were cooled for 15 min on ice and were aspirated dry prior to adding T3D-MAB complexes prepared by incubating 3.3 $\times$ 10⁷ PFU of T3D per ml in MEM⁺ with an equal volume of MAB (1.0 to 1,000 μg of MAB per ml, diluted in MEM⁺) for 1 h on ice. One hundred fifty microliters per well of T3D-MAB complexes was added to cells (multiplicity of infection, $\sim$ 5), followed by incubation for 1 h on ice. Unbound T3D-MAB complexes were removed by adding 1.0 ml of ice-cold phosphate-buffered saline (PBS) and aspirating the well dry prior to adding 0.5 ml of MEM⁺. Some samples were immediately frozen to determine the amount of virus present at the start of the growth experiment ($t = 0$). Other clusters were incubated (37°C, 5% CO₂) for 24 h to allow viral replication prior to freezing. Virus was titered after three freeze-thaw cycles. Data for both $t = 0$ and $t = 24$ h were obtained from triplicate wells. Data are presented from four separate experiments, each datum point at a single MAB concentration. Experiments were repeated for MABs which inhibited growth over a narrow concentration range (μ 1-8H6 and σ 3-4F2) with results comparable to those shown. Log titers from triplicate wells did not vary by more than 5%, and therefore data are presented without error bars. We calculated the PFU yield ($t = 24$ h) per input PFU ($t = 0$ h) by dividing the titer at $t = 24$ by the titer at $t = 0$. To normalize for differences in control yield between different experiments, data for different MABs are presented as the percent yield seen with control MAB σ 1T1L-5C6. Control yield ranged from 275 to 1,380 PFU per input PFU.

Effect of MABs on sensitivity of T3D replication to ammonium chloride. This experiment was performed as for single-cycle T3D growth, with the following alterations. T3D-MAB complexes were prepared by using MABs at 40 $\mu\text{g/ml}$. After T3D-MAB complexes were bound to cells on ice and unbound T3D-MAB complexes were washed away, 1 ml of prewarmed MEM⁺ was added to each well prior to moving to 37°C and 5% CO₂. At 0, 30, and 120 min of incubation at 37°C, 1 ml of MEM⁺ containing 40 mM NH₄Cl was added to a set of wells. Viral growth was for 24 or 48 h. Data from triplicate wells are presented as mean log titers without error bars, as log titers did not differ by more than 5% within any set of triplicate wells.

Binding of T3D to L cells. L cells (5 $\times$ 10⁴ per well) were plated in 96-well flat-bottomed plates (Costar) and were incubated overnight at 37°C in 5% CO₂. ³⁵S-T3D was diluted to 0.44 mg/ml in HBSS⁺ and was precleared of aggregates by centrifugation at 4,930 $\times$ g for 5 min (Eppendorf 5415C Microfuge) (precleared ³⁵S-T3D). Virus was mixed 1:1 with MABs (1.0 mg/ml in PBS) and was incubated on ice for 1 h. Forty microliters of T3D-MAB complex per well was added in triplicate to prechilled L cells from which medium had been removed; this was followed by incubation for 1 h on ice with rocking every 15 min. Wells were washed twice with 200 μl of HBSS⁺ to remove unbound T3D-MAB complexes. Cells and bound virus were released from wells by incubation in 50 μl of trypsin-EDTA (Biofluids) (37°C for 5 min) and were counted. The amount of virus specifically bound to cells was calculated by subtracting cpm bound to wells containing no cells from cpm bound to wells containing cells. Percent binding inhibition was calculated by comparing cpm bound in the presence of a test MAB with cpm bound in the presence of control MAB σ 1T1L-5C6. For studies of binding of T3D- σ 3-10G10 complexes to L cells, the same protocol was followed except that σ 3-10G10 (1.0 mg/ml) was mixed with precleared ³⁵S-T3D at 0.44 mg/ml for 1 h on ice, and then aliquots of this mixture were further incubated with an equal volume of either σ 1T3D-G5 or control MAB σ 1T1L-5C6 (each at 1.0 mg/ml) for 1 h on ice prior to the addition to L cells. Binding experiments

were performed 2 to 3 times for each MAb, and data are presented as mean percent inhibition of binding $\pm$ the standard error of the mean.

Antibody effects on proteolytic uncoating of T3D by L cells. L cells (1.4×10^6 per well) were plated overnight in MEM⁺ in 6-well clusters (Costar), placed on ice for 1 h, and washed three times with ice-cold HBSS⁺ to remove unbound cells. Wells were aspirated dry, and 400 μ l of HBSS⁺ and 100 μ l of T3D-MAB complex were added. T3D-MAB complexes were prepared by incubating 0.25 to 0.44 mg of ³⁵S-T3D per ml with an equal volume of MAb (0.1 to 1,000 μ g/ml in HBSS⁺) on ice for 1 h. Cells were incubated with T3D-MAB complexes for 2 h on ice with intermittent rocking. Unbound T3D-MAB complexes were removed by washing three times with ice-cold HBSS⁺. Three milliliters of RPMI⁺ was then added, and clusters were incubated for 2 h at 37°C to allow internalization and proteolytic uncoating of labelled virus. Clusters were put on ice and washed three times with ice-cold HBSS⁺. Medium was removed and replaced with 200 to 400 μ l of cell lysis buffer (0.01 M Tris, 0.25 M NaCl, 5 mM MgCl₂, 0.5% Nonidet P-40, 0.1% Tween 20, 10 mM β -mercaptoethanol, 5 μ g of leupeptin per ml, 5 μ g of pepstatin per ml, 1% aprotinin, and 2.5 mM phenylmethylsulfonyl fluoride [protease inhibitors from Sigma, St. Louis, Mo.]). Cells were scraped off the well and were transferred to tubes containing 1/4 sample volume of 4 $\times$ Laemmli sample buffer (containing β -mercaptoethanol), boiled for 5 min, and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Autoradiograms were quantitated by using a Dage-MTI CCD72 camera and a Matrox MVP image processing board with FL4000 software from Georgia Instruments (Roswell, Ga.). The extent of uncoating was quantitated by dividing the amount of μ 1 present as the processed δ fragment by the total amount of μ 1 present (μ 1C + δ). Values for individual MAbs in different experiments were normalized and expressed as percent control uncoating, by using uncoating in the presence of control MAbs σ 1T1L-5C6 or μ 1-10H2 (35) (which did not alter processing) as 100%.

Internalization of T3D by L cells. Precleared ³⁵S-T3D was diluted to either 0.44 mg/ml or 0.09 mg/ml in HBSS⁺. Results of experiments using these two virus concentrations were similar, and data were therefore pooled from 2 to 3 different experiments for each MAb. Equal volumes of precleared ³⁵S-T3D and MAbs at 1.0 mg/ml were incubated on ice for 1 to 2 h and were then microcentrifuged at $2,515 \times g$ to remove virus-antibody complexes. Recovery after centrifugation was $101 \pm 0.5\%$ for σ 1-T1L 5C6, $94 \pm 2.2\%$ for σ 3-10G10, $42 \pm 2.5\%$ for σ 3-4F2, and $98 \pm 4\%$ for μ 1-10F6. L cells were plated and washed as for assessment of proteolytic uncoating of T3D above. Wells were aspirated dry, and 400 μ l of HBSS⁺ and 100 μ l of T3D-MAB complex was added. Plates were incubated for 1 h on ice, washed three times with HBSS⁺, and aspirated dry, and then 3 ml per well of RPMI⁺ (37°C, 5% CO₂) or HBSS⁺ (on ice, room air) was added. Plates were either incubated at 37°C in 5% CO₂ for 30 or 60 min to allow internalization of bound virus or were kept on ice for 60 min (no internalization control). Internalization was stopped by transferring plates to ice. Wells were washed three times with PBS-0.5 mM EDTA and were aspirated dry, and virus on the cell surface was removed by proteolysis (20 min, room temperature) by using 400 μ l per well of trypsin-EDTA (Biofluids) containing 25 μ g of chymotrypsin (Sigma) per ml, 50 μ g of proteinase K per ml (Boehringer Mannheim, Indianapolis, Ind.) and 20 mM HEPES. Proteolysis was stopped by transfer to ice and the addition of 100 μ l per well of FCS containing 0.1 mg of trypsin inhibitor per ml (Sigma), 12.5 mM phenylmethylsulfonyl fluo-

ride, 50 mM EDTA, and 10% aprotinin (Sigma). Cells and medium containing released virus were separated by centrifugation ($2,515 \times g$, 5 min) in microcentrifuge tubes containing 0.5 ml of 50% dibutyl phthalate-50% bis(2-ethylhexyl)phthalate (Kodak, Rochester, N.Y.). Cells and internalized virus pellet through the phthalate cushion and are therefore separated from medium containing released virus. At the end of this procedure, >90% of the cells excluded trypan blue (data not shown). Aliquots of released virus and cell pellet were counted. The percent virus internalized (protease resistant) was calculated by dividing cpm in the cell pellet by total cpm recovered (cpm in pellet + cpm released) and then subtracting the percentage of counts remaining cell associated when cells were incubated at 4°C for 60 min (4% for σ 1T1L-5C6, $5.3 \pm 2\%$ for σ 3-10G10, $29.5 \pm 5.5\%$ for σ 3-4F2, and $4.5 \pm 0.5\%$ for μ 1-10F6). Conditions used here for proteolysis were harsher than those used by Strong et al. (29), because virus-antibody complexes are more difficult to remove from the cell surface than is free virus (data not shown). Data are presented as the mean percent internalized $\pm$ standard deviation.

Immunoprecipitation of T3D. Fifty microliters of precleared ³⁵S-T3D diluted to 0.44 mg/ml in HBSS⁺ was mixed with 50 μ l of serial 10-fold dilutions (0.1 to 1,000 μ g/ml) of MAbs in HBSS⁺ and was incubated on ice for 1 h. Samples were centrifuged at $2,515 \times g$ for 5 min to pellet T3D-MAB complexes, and samples of the supernatant were counted to determine the amount of virus remaining in solution. We evaluated the size of reovirus aggregates pelleted by this procedure by using reovirus aggregates made with phenol or ethanol (7). Phenol treatment generates aggregates containing 75 ± 25 virions, while ethanol treatment generates aggregates containing 250 ± 100 virions. Centrifugation at $2,515 \times g$ for 5 min quantitatively pelleted both phenol and ethanol-generated aggregates (data not shown), demonstrating that virus remaining in the supernatant after centrifugation is present in aggregates no larger than 75 to 250 virions. The percent virus remaining in the supernatant was calculated by dividing cpm in the supernatant after centrifugation by cpm present prior to centrifugation. Immunoprecipitation experiments were performed at least twice with similar results, and data from a single experiment are presented.

In vitro proteolytic uncoating of T3D. Chymotrypsin (Sigma) was prepared as a 2.5-mg/ml stock in dialysis buffer and was frozen at -20°C until use. The effect of antibody on chymotrypsin digestion of T3D was assessed by incubating 20 μ l of either 0.11 or 0.33 mg of ³⁵S-T3D per ml with 10 μ l of 1.0 mg of MAb per ml for 1 h on ice. A total of 3.6 μ l of chymotrypsin stock (or a 1:5 dilution of chymotrypsin stock in dialysis buffer) was added, and the mixture was incubated for 5 min in a 37°C water bath. Digestion was stopped by adding 15 μ l of 10 mM phenylmethylsulfonyl fluoride (in PBS) and placing the mixture on ice. Fifteen microliters of 4 $\times$ Laemmli sample buffer (containing β -mercaptoethanol) was added, and samples were boiled for 2 min; this was followed by 10% SDS-PAGE and autoradiography.

RESULTS

Inhibition of T3D binding to L cells. The initial step in reovirus infection of target cells is binding to cell surface receptors. To assess effects of reovirus-specific MAbs on this step in infection, we incubated MAbs at 500 μ g/ml with ³⁵S-T3D and then compared binding of virus-MAB mixtures with that of virus alone. Binding reactions were performed at 4°C to prevent internalization of virus. This MAB concentration was selected to enhance chances of detecting effects of

FIG. 1. Effects of MAbs at 500 $\mu\text{g/ml}$ on binding of T3D to L cells. Data are presented as the percent control binding, with 100% being the amount of ^{35}S -T3D bound in the presence of control MAb $\sigma 1\text{T1L-5C6}$. Data are presented as the means of two to three experiments each $\pm$ the standard error of the mean. Protective and nonprotective refer to prevention of lethal infection of neonatal mice (Table 1).

MAbs on binding to cells and represents ~ 800 MAb molecules per virion particle. As each virion has 600 copies of $\sigma 3$ and $\mu 1$ (8), this represents about one MAb molecule per $\sigma 3$ or $\mu 1$ protein on the virion. As expected, a MAb specific for the $\sigma 1$ cell attachment protein ($\sigma 1\text{T3D-G5}$) inhibited $>90\%$ of T3D binding to L cells and was used as a positive control (Fig. 1). $\sigma 3$ and $\mu 1$ MAbs produced minimal ($<20\%$) or no inhibition of viral binding to L cells, even with MAbs present at high concentrations. There was no correlation between capacity of MAbs to protect mice against lethal challenge with T3D and binding inhibition (Table 1, Fig. 1).

Since the capacity of MAbs to inhibit viral binding *in vitro* did not correlate with their capacity to protect *in vivo*, we next considered the possibility that MAbs act at steps in T3D replication subsequent to viral binding to target cell receptors. Experiments designed to evaluate postbinding effects of MAbs were performed by preincubating virus plus MAb, allowing binding of T3D-MAB complexes to cells at 4°C , and then removing all unbound MAB and virus by washing. By following the fate of virus-MAB complexes bound to the cell surface, we defined effects of MAbs on virus replication independent of effects on binding.

Effects of MAbs on T3D growth. To assess the growth capacity of MAB-virus complexes bound to the cell surface, we performed the following experiment. T3D-MAB complexes were bound to L cells at 4°C , unbound virus and MAB were removed by washing, and cells were either frozen immediately ($t = 0$) or incubated for 24 h at 37°C to allow viral growth. Viral titer was determined and the yield per input PFU was determined by using growth in the presence of a control MAB ($\sigma 1\text{T1L-5C6}$) as a reference (Fig. 2). Viral yield reflects the increase in viral titer at 24 h compared with the amount of virus bound to cells at $t = 0$ and thus controls for MAB effects on initial viral binding. Three protective $\sigma 3$ MAbs ($\sigma 3$ -4F2, $\sigma 3$ -10C1, and $\sigma 3$ -10G10) and the protective $\mu 1$ MAB $\mu 1$ -8H6 inhibited viral growth (Fig. 2) by at least 90% at 500 $\mu\text{g/ml}$. Nonprotective MAbs $\sigma 3$ -8F12 and $\mu 1$ -10F6 had no effect on viral growth. MAbs which inhibited growth did not efficiently decrease plaque number in neutralization assays (Table 1). However, inhibition of growth correlated with the capacity of MAbs to produce small plaques in these assays (compare Table 1 and Fig. 2).

At a MAB concentration of 500 $\mu\text{g/ml}$ there are (assuming a particle-to-PFU ratio of 100) approximately 1.2×10^6 MAB molecules per virion and 2,000 molecules per target $\sigma 3$ or $\mu 1$

FIG. 2. Effects of MAbs on single-cycle growth of T3D. MAbs at varying concentrations were mixed with T3D at 4°C , and resulting MAB-T3D complexes were bound to L cells at 4°C . Unbound complexes were washed away, and titers were determined either immediately ($t = 0$) or after 24 h of replication ($t = 24$). The titers of T3D were determined from triplicate wells at each antibody concentration at both $t = 0$ and $t = 24$. The yield in the presence of test MAbs was calculated as $\text{PFU } t = 24 / \text{PFU } t = 0$. The percent control yield was calculated as $(\text{yield in the presence of test MAB}) / (\text{yield in the presence of control MAB } \sigma 1\text{T1L-5C6}) \times 100$. Protective and nonprotective refer to prevention of lethal infection of neonatal mice (Table 1).

protein on the virion. MAB $\sigma 3$ -10G10 inhibited growth by 90% at a concentration of 0.5 $\mu\text{g/ml}$, which translates to 1.2×10^3 MAB molecules per virion or two MAB molecules per target $\sigma 3$ protein. Further evidence that inhibition of growth was not due to inhibition of binding comes from the fact that $\sigma 3$ -10C1 and $\sigma 3$ -10G10 decreased viral yield $>99\%$ at concentrations (50 to 500 $\mu\text{g/ml}$) that produced only minimal ($<20\%$) inhibition of binding (Fig. 1). Growth inhibition is not strictly analogous to neutralization as defined in standard plaque reduction assays, since $\sigma 3$ -10C1 inhibited growth significantly at 0.5 $\mu\text{g/ml}$ and yet is nonneutralizing at concentrations as high as 100 $\mu\text{g/ml}$ (Table 1). These results suggested that the best *in vitro* correlate of *in vivo* protection was the capacity of MAbs to inhibit viral growth and that this effect occurred as a result of MAB inhibition of reovirus replication at a step or steps subsequent to viral binding.

MAbs inhibit early steps in reovirus infection of the cell: MAB effects on T3D sensitivity to ammonium chloride. Following internalization, virions undergo proteolytic uncoating generating ISVPs in an acid intracellular compartment (introduction). Thus, growth of intact virions is inhibited by ammonium chloride, while growth of ISVPs is not. If $\sigma 3$ MAbs inhibit or delay events in T3D replication prior to ISVP formation, they should prolong the time that T3D growth is sensitive to inhibition by ammonium chloride. To test this possibility, T3D-MAB complexes were bound to cells, unbound virus and MAB were removed, cells were warmed to 37°C , and ammonium chloride was added at varying times. In the presence of control MAB ($\sigma 1\text{T1L-5C6}$), the addition of ammonium chloride at $t = 0$ produced nearly complete inhibition of viral growth. By 30 min postinfection a significant fraction of virus had escaped the ammonium chloride-sensitive step, and by 120 min postinfection virus infection was not altered by the addition of ammonium chloride (Fig. 3A). Growth of T3D coated with $\sigma 3$ -10G10 was completely inhibited by the addition of

FIG. 3. Effect of σ 3-10G10 on the duration of T3D susceptibility to growth inhibition by ammonium chloride. Control MAb σ 1T1L-5C6-treated T3D (A) or T3D- σ 3-10G10 complexes (B) were bound to L cells, and unbound complexes were washed away. At different times ($t = 0, 30$, and 120 min) after cells were warmed to 37°C , medium containing ammonium chloride (final concentration, 20 mM) was added. Replication was assessed at 24 and 48 h. Data are presented as the mean log titers of triplicate wells.

ammonium chloride at both $t = 0$ and $t = 30$ min (Fig. 3B). By $t = 120$, σ 3-10G10-coated T3D was relatively insensitive to growth inhibition by ammonium chloride. This result suggested that σ 3 MAb inhibit a step in viral replication prior to the successful generation of ISVPs.

MAbs inhibit early steps in reovirus infection of the cell: MAb effects on proteolytic uncoating of T3D by L cells. A critical early step in reovirus infection of the cell is proteolytic uncoating of the virion. To further assess effects of MAbs on early steps leading to proteolytic uncoating, ^{35}S -T3D-MAB complexes (prepared with varying amounts of MAb) were bound to cells at 4°C , unbound virus and MAb were removed, and cells were warmed to allow uncoating (Fig. 4). Proteolytic uncoating was quantitated by autoradiographic assessment of μ 1C cleavage to its δ fragment (Fig. 4A). At high concentrations (500 $\mu\text{g/ml}$) of control MAb (σ 1T1L-5C6), all μ 1 MAbs, and the nonprotective MAb σ 3-8F12, no inhibition of capsid proteolysis was observed (Fig. 4A). Protective MAbs σ 3-10C1, σ 3-4F2, and σ 3-10G10 inhibited uncoating of T3D at high concentrations (Fig. 4A) as well as concentrations as low as 5 $\mu\text{g/ml}$ (Fig. 4B). These results confirmed that σ 3 MABs inhibit critical early events in T3D infection. Together with the ammonium chloride experiment described above, these results demonstrated that the critical step(s) inhibited by protective MAbs occurs before or during intracellular proteolytic uncoating of the virion.

Effect of MAbs on internalization of virus. Having shown that σ 3 MABs inhibit early postbinding events in reovirus infection, we proceeded to analyze MAB effects on sequential early steps in infection of cells. We first examined MAB effects on virion internalization, a process which occurs at 37°C but not 4°C . T3D-MAB complexes on the cell surface are susceptible to removal by extracellular proteases, whereas internalized virions are not. Thus, inhibition of internalization of T3D-MAB complexes would be expected to result in prolonged susceptibility of virus-MAB complexes to proteolytic removal from the cell surface. We therefore assessed the capacity of protease treatment to remove ^{35}S -T3D-MAB complexes from the cell surface at sequential times following warming to 37°C (Fig. 5A). σ 1-10G10 inhibited internalization by $\sim 50\%$ 30 and 60 min after warming cells to 37°C ; σ 3-4F2 was more effective

than σ 3-10G10. Control MAb (σ 1T1L-5C6) and nonprotective MAb μ 1-10F6 did not alter internalization.

We considered the possibility that inhibition of internalization was due to σ 3-MAB-mediated alteration of the mechanism by which virions bind to the cell surface. However, MAb σ 1T3D-G5, which specifically interacts with the cell attachment protein σ 1, inhibited binding of σ 3-10G10-T3D complexes to cells by 98% ($n = 2$ experiments). This indicates that attachment of these complexes continues to occur in the usual manner via the cell attachment protein σ 1.

Inhibition of virion internalization could also result from MAB-induced cross-linking of virions into immune complexes large enough to be poorly internalized by L cells. We therefore examined the capacity of MAbs at varying concentrations to immunoprecipitate virions. ^{35}S -T3D was incubated with MAbs at varying concentrations, and the resulting mixture was then microcentrifuged to pellet immune complexes (Fig. 6). We found that σ 3-10C1, σ 3-10G10, and σ 3-4F2 all produced dose-dependent immunoprecipitation of T3D. Both MAb σ 3-10G10 and MAb σ 3-10C1 exhibited typical zones of antibody excess and antigen excess. The ratio of MAB (at 500 $\mu\text{g/ml}$) to virus particle in the zone of antibody excess was the same as that for binding inhibition experiments (~ 800 MAbs per virion and ~ 1 MAB per target σ 3 or μ 1 molecule).

MAB effects on intracellular proteolytic uncoating of T3D. Since σ 3-10G10 inhibited both viral internalization (Fig. 5A) and proteolytic uncoating of the virion (Fig. 4), we wished to determine whether these effects could occur independently. Although σ 3-10G10 inhibited viral internalization at 30 and 60 min (Fig. 5), this effect was incomplete. Some σ 3-10G10-T3D complexes were internalized as early as 15 min (data not shown), and the amount of σ 3-10G10-T3D complex internalized increased with time (Fig. 5A). The magnitude of σ 3-10G10-mediated inhibition of proteolytic uncoating (>80 to 90% inhibition after 2 h at 37°C [Fig. 4]) was far greater than its effects on internalization, and thus inhibition of internalization did not completely explain effects of σ 3-10G10 on early events in T3D infection. We therefore removed T3D- σ 3-10G10 complexes from the surface of cells after 60 min at 37°C and evaluated the extent of proteolytic uncoating of internalized σ 3-10G10-T3D complexes (Fig. 5B). This analysis was

FIG. 4. Effects of MAbs on proteolytic uncoating of T3D by L cells. ^{35}S -T3D-MAB complexes were bound to L cells at 4°C , and unbound complexes were washed away. Cells were warmed to 37°C for 2 h, and virus was harvested and subjected to SDS-PAGE and autoradiography. (A) Autoradiogram showing T3D protein profiles after incubation of virus-MAB (500 $\mu\text{g}/\text{ml}$) complexes with L cells for 2 h at 37°C . The percent of total $\mu 1$ protein ($\mu 1\text{C} + \delta$) present as the δ fragment is shown below each lane. Lane T3D at far right shows the autoradiographic profile of ^{35}S -labelled T3D proteins prior to exposure to L cells. (B) Autoradiographic quantitation of the extent of proteolytic uncoating of T3D-MAB complexes prepared at different MAB concentrations. Uncoating was quantitated as the proportion of $\mu 1$ present as the δ fragment divided by the total $\mu 1$ recovered in a sample ($\delta/\mu 1\text{C} + \delta$). Percent control uncoating was calculated as (uncoating in the presence of test MAB)/(uncoating in the presence of control MAB $\sigma 1\text{-T1L-5C65}$) $\times 100$. The ratio of $\delta/\mu 1\text{C} + \delta$ present in the starting purified ^{35}S -T3D was also quantitated for each experiment and is shown as unprocessed T3D.

possible because we could quantitatively remove T3D- $\sigma 3$ -10G10 complexes from the cell surface (Materials and Methods) and thus visualize the fate of internalized T3D- $\sigma 3$ -10G10 complexes by using SDS-PAGE. $\sigma 3$ -10G10 (but not control MAB $\sigma 1\text{-T1L-5C6}$ or nonprotective MAB $\mu 1$ -10F6) inhibited proteolytic uncoating of internalized T3D, as shown by the lack of processing of $\mu 1\text{c}$ to the δ fragment (Fig. 5B). These results showed that MAbs can directly inhibit intracellular proteolytic uncoating of virions and that this occurs independent of inhibition of internalization.

Effect of MAbs on proteolysis of T3D in a cell-free system. One mechanism which would explain MAB-mediated inhibi-

FIG. 5. Internalization of T3D by L cells and extent of proteolytic uncoating of internalized T3D in the presence of different MAbs. Virus-MAB complexes were bound to L cells, unbound complexes were removed by washing, and the cells were warmed to allow internalization and uncoating. At different times, the amount of virus internalized was quantitated, and the extent of uncoating of internalized virus was determined. (A) Graph of the percent of reovirus internalized (not removed from the cell surface by proteolysis) after incubation of cells for 30 or 60 min at 37°C in the presence of different MAbs. (B) Autoradiogram showing the extent of uncoating of reovirus internalized (i.e., remaining cell associated after removal of cell surface virus by proteases) after 60 min at 37°C .

tion of intracellular proteolytic uncoating was direct MAB-mediated protection of capsid proteins from intracellular proteases. Digestion of virions *in vitro* by chymotrypsin results in ISVPs structurally and functionally indistinguishable from cell-generated ISVPs (introduction) and thus provided a model in which we could directly assess the effects of MAbs on capsid protein proteolysis.

MAbs $\sigma 3$ -10G10 and $\sigma 3$ -10C1 inhibited chymotrypsin-mediated uncoating of T3D (Fig. 7A, compare lanes 7 and 8 with lanes 2 to 6) as well as proteolytic uncoating of virus in L cells (Fig. 4). The specificity of this inhibition is demonstrated by the fact that other MAbs specific for $\sigma 3$ ($\sigma 3$ -8F12, Fig. 7A, lane 5, and $\sigma 3$ -4F2, Fig. 7A, lane 6), $\mu 1$ ($\mu 1$ -8H6, Fig. 7A, lane 4, and $\mu 1$ -10F6 [data not shown]), or $\sigma 1$ ($\sigma 1\text{-T3D-G5}$ [data not shown]) did not inhibit chymotrypsin-induced uncoating. Protective MAB $\sigma 3$ -4F2 did not inhibit uncoating of T3D by

FIG. 6. Immunoprecipitation of purified T3D by MAbs. MAbs at different concentrations were mixed with ^{35}S -T3D, incubated for 1 h on ice, and then centrifuged. Data are presented as the percentage of counts remaining in the supernatant after centrifugation. Immunoprecipitation profiles were repeated at least twice with each MAb, and a representative single experiment is presented. The unusual concentration dependence for immunoprecipitation by MAb σ 3-4F2 is unexplained but was consistent.

chymotrypsin but did inhibit proteolytic uncoating of virus by L cells (Fig. 4). μ 1 MAbs (including protective MAb μ 1-8H6) failed to inhibit both chymotrypsin-mediated uncoating of T3D (Fig. 7A) and proteolytic uncoating of virus in L cells (Fig. 4).

MAb-mediated inhibition of σ 3 cleavage by chymotrypsin generated two patterns of σ 3 breakdown (Fig. 7A and B). The first pattern (σ 3-10G10, Fig. 7A, lane 7, and Fig. 7B, lane 4) showed protection of intact σ 3 and decreased cleavage of μ 1c to δ . The second pattern (σ 3-10C1, Fig. 7A, lane 8, and Fig. 7B, lane 5) showed protection of fragments of σ 3 rather than the full-size protein. σ 3-10C1-protected fragments of σ 3 (Fig. 7B), even when the chymotrypsin concentration was increased fivefold and viral concentration was decreased threefold rela-

tive to concentrations shown in Fig. 7A. Despite the high concentration of chymotrypsin in this experiment (Fig. 7B), protection of fragments of σ 3 was accompanied by complete inhibition of cleavage of μ 1C to δ (compare Fig. 7B, lane 5, with lanes 2 to 4). These experiments showed that certain σ 3 MAbs directly inhibit steps in proteolysis of reovirus outer capsid proteins which lead to uncoating. MAbs inhibited proteolysis of σ 3 by different mechanisms, accounting for differences in efficiency of protection of full-size σ 3 versus fragments of σ 3. Inhibition of σ 3 proteolysis was always associated with decreased cleavage of μ 1C to δ .

DISCUSSION

In this paper we describe the mechanisms by which protective MAbs specific for the reovirus outer capsid protein σ 3 act at the level of the cell to inhibit viral replication. We recently characterized these MAbs and evaluated their capacity to protect neonatal mice from lethal infection (32, 35). The capacity of MAbs to protect mice did not correlate completely with MAb properties including isotype, avidity, plaque reduction neutralization, and hemagglutination inhibition. To identify mechanisms of in vitro antibody action that correlate with in vivo protection, we studied MAb effects on early steps in reovirus replication in L cells. Our findings are summarized in Table 2 and Fig. 8.

Protective σ 3 and μ 1 MAbs did not significantly inhibit reovirus binding to target cells, while MAb specific for the viral cell attachment protein σ 1 did inhibit binding (Fig. 1 and 8, step A). Protective MAbs, but not their nonprotective counterparts, inhibited viral growth independent of effects on binding (Fig. 2). Protective σ 3 MAbs act by inhibiting early steps in viral replication in L cells (Fig. 3 and 4). Analysis of σ 3 MAb effects on postbinding steps in reovirus replication identified two specific early events inhibited by these MAbs: (i) internalization of virus bound to the cell surface (Fig. 5A and 8, step B) and (ii) intracellular proteolytic uncoating of virions (Fig. 4, 5B, and 8, step C). One likely explanation for the effects of MAbs on intracellular proteolytic uncoating is direct MAb-mediated inhibition of the action of proteases on viral capsid proteins (Fig. 7).

FIG. 7. Effect of MAbs on digestion of ^{35}S -T3D by chymotrypsin in vitro. ^{35}S -T3D was incubated with MAbs at 333 $\mu\text{g}/\text{ml}$ and was then digested with chymotrypsin at 37°C for 5 min. Designations of different reovirus proteins and protein size classes are shown at the left and right of autoradiograms. Designations and specificities of MAbs as well as whether chymotrypsin was added to a sample are indicated at the top. (A) Digestion of 0.22 mg of ^{35}S -T3D per ml with 54 μg of chymotrypsin per ml for 5 min at 37°C. (B) Digestion of 0.07 mg of ^{35}S -T3D per ml with 270 μg of chymotrypsin per ml for 5 min.

TABLE 2. Effects of MAbs on reovirus infection of L cells

MAb	Inhibition of ^a						Protects in vivo ^b
	Binding to L cells ^c	Growth in L cells ^d	Uncoating by L cells ^e	Internalization ^f	Uncoating inside L cells ^g	Chymotrypsin digestion ^h	
σ 1T3D-G5	Yes						Yes
μ 1-10F6	No	No	No	No	No	No	No
μ 1-8H6	No	Yes	No			No	Yes
σ 3-8F12	No	No	No			No	No
σ 3-4F2	No	Yes	Yes	Yes		No	Yes
σ 3-10C1	No	Yes	Yes			Yes	Yes
σ 3-10G10	No	Yes	Yes	Yes	Yes	Yes	Yes

^a Blanks indicate not done.^b See Table 1.^c See Fig. 1.^d See Fig. 2.^e See Fig. 4.^f See Fig. 5A.^g See Fig. 5B.^h See Fig. 7.

σ 3-specific MAbs inhibit reovirus replication independent of effects on binding of virus to the cell by inhibiting early steps in infection of the cell. Protective MAbs, but not their nonprotective counterparts, decreased viral yield per input PFU at 24 h by up to 99% in a growth assay (Fig. 2). This growth experiment was designed to eliminate any effect of MAbs on the efficiency of binding of virus to the cell. In addition, protective σ 3 and μ 1 MAbs had minimal effects on binding of virus to the cell (Fig. 1). Therefore protective MAbs can act to inhibit replication independent of MAb effects on binding of virus to the cell. This represents the first consistent in vitro correlate of antibody-mediated protection in vivo that we have been able to identify (see reference 32). Since protection did not correlate with or involve binding inhibition, protective MAbs must inhibit steps in reovirus replication that occur subsequent to virus attachment to the cell. These considerations do not diminish the biologic importance of binding inhibition by some MAbs (Fig. 8, step A). For example, MAb σ 1T3D-G5 inhibits binding of virus to cells (e.g., Fig. 1) and is protective in vivo (32–34).

We performed two experiments to define whether early events in reovirus infection were the target(s) of protective

σ 3-MAb-mediated inhibition of viral growth. Ammonium chloride blocks pH-dependent intraendosomal proteolytic uncoating of reovirus (30). Inhibition of steps in replication up to or including proteolytic uncoating should increase the time during which virus replication remains sensitive to inhibition by ammonium chloride. MAb σ 3-10G10 significantly prolonged the duration and extent of T3D sensitivity to ammonium chloride (Fig. 3), indicating that protective σ 3 MAbs can act at or prior to intracellular proteolytic uncoating of reovirus. This was independently confirmed by the direct demonstration that several protective σ 3 MAbs inhibited proteolytic uncoating of reovirus by L cells (Fig. 4). These experiments showed that protective σ 3 MAbs target steps which occur after virus is bound to the cell and at or before proteolytic uncoating of the virus.

It is important to note that protective MAb μ 1-8H6 acts at some postbinding step to inhibit reovirus growth (Fig. 1 and 2) but that we did not define the precise step(s) at which this protective MAb acts. This antibody had no effect on proteolytic uncoating of virions by cells under conditions used in this study (Fig. 4) and did not inhibit in vitro uncoating of the virus by chymotrypsin (Fig. 7A). Thus μ 1-8H6 is less effective than are

FIG. 8. Three steps at which antibodies can act to block reovirus replication.

the three protective $\sigma 3$ MAbs analyzed here, either at inhibiting early steps in reovirus infection or at inhibiting direct interactions of proteases with the capsid. Together, these results suggest that MAb $\mu 1$ -8H6 acts to inhibit viral replication at a step after intracellular proteolytic uncoating of the virus (Fig. 8, step C). One potential target for this $\mu 1$ MAb is penetration of intracellular membranes after generation of ISVPs by intracellular proteases. This step is critical for virus replication and likely involves the $\mu 1$ protein to which $\mu 1$ -8H6 binds (18, 20, 31). It is interesting to note that $\mu 1$ -8H6 does bind ISVPs and that it recognizes the δ fragment of $\mu 1C$ and thus might stably associate with virus throughout the process of viral entry into the cell and initial uncoating of the virion (35). It is possible therefore that $\mu 1$ -8H6 could directly inhibit interaction of $\mu 1$ -derived polypeptides with vesicular membranes or could block conformational changes which occur during the formation of ISVPs which are critical for membrane penetration (8).

MAb effects on internalization. Following binding to cell surface receptors, reovirus is internalized and appears within endocytic vesicles (Fig. 8, step B). Inhibition of virion internalization provides a potential early step in reovirus replication at which $\sigma 3$ MAbs could act to inhibit viral growth. Two protective MAbs, $\sigma 3$ -10G10 and $\sigma 3$ -4F2, inhibited internalization (Fig. 5A and Table 2). Nonprotective $\mu 1$ -MAb 10F6 did not inhibit internalization, demonstrating that inhibition of internalization is not due simply to the presence of MAb on the virion surface. While multiple mechanisms of action likely contribute to growth inhibition by $\sigma 3$ -10G10 (see below), inhibition of internalization is likely the dominant postbinding mechanism of action for protective MAb $\sigma 3$ -4F2. $\sigma 3$ -4F2 inhibits growth independent of its effects on binding and inhibits internalization, but it did not inhibit *in vitro* proteolytic uncoating of the virion by chymotrypsin. This suggests that $\sigma 3$ -4F2-mediated inhibition of proteolytic uncoating by L cells (Fig. 4) results from inhibition of virion internalization rather than from direct blockade of protease action on $\sigma 3$. Blockade of internalization would prevent virus from entering into the intracellular endosomal compartment in which capsid proteolysis occurs (Fig. 8, step B). The failure of $\sigma 3$ -4F2 to inhibit proteolytic uncoating of virions by chymotrypsin is consistent with the fact that its epitope on $\sigma 3$ is distinct from that of both $\sigma 3$ -10G10 and $\sigma 3$ -10C1 (35), both of which do block chymotrypsin digestion. This suggests that MAbs specific for different epitopes on a single capsid protein can inhibit the same step in viral replication (intracellular proteolytic uncoating) but that they do so by different mechanisms.

The mechanism(s) by which MAbs $\sigma 3$ -4F2 and $\sigma 3$ -10G10 inhibit internalization of bound virions is unknown. Both $\sigma 3$ -4F2 and $\sigma 3$ -10G10 cross-link virions into aggregates (Fig. 6). It is possible that these aggregates are too large to be effectively internalized by L cells. Cross-linking of virus on the cell surface could be an important mechanism *in vivo* if entry into the cell and therefore viral replication are blocked. In our experiments, MAb concentrations required to inhibit growth or block proteolytic uncoating of the virion by L cells ranged, depending on the assay, from 0.5 to 500 $\mu\text{g}/\text{ml}$. For example, $\sigma 3$ -MAb 4F2 immunoprecipitated virions at concentrations between 5 and 500 $\mu\text{g}/\text{ml}$, which correlated well with the concentrations of $\sigma 3$ -4F2 required to inhibit proteolytic uncoating of the virion capsid by L cells (compare Fig. 4 and 6). If one assumes that 5% of serum immunoglobulin G is virus specific at the peak of a response and that the concentration of immunoglobulin G in mouse serum is about 10 mg/ml , concentrations of specific polyvalent immunoglobulin G *in vivo* are $\sim 500 \mu\text{g}/\text{ml}$ (13). Thus, concentrations of antibody *in vivo*

may well be sufficient to cross-link virus on the cell surface or to generate poorly internalized aggregates in the circulation.

MAb $\sigma 3$ -10G10 inhibited internalization at 500 $\mu\text{g}/\text{ml}$, which corresponds to a condition of antibody excess (Fig. 6), in which large aggregates of virus should not occur. In addition, internalization experiments were carried out with virus-MAb mixtures cleared of large aggregates by centrifugation (Materials and Methods). This suggests that $\sigma 3$ -10G10-mediated inhibition of internalization is not due to viral aggregation. For both $\sigma 3$ -10G10 and $\sigma 3$ -10C1, the ratio of MAb (at 500 $\mu\text{g}/\text{ml}$) to $\sigma 3$ target in growth inhibition experiments (~ 2000 ; Fig. 2) is much higher than the ratio of MAb (at 500 $\mu\text{g}/\text{ml}$) to $\sigma 3$ target in immunoprecipitation, internalization ($\sigma 3$ -10G10 only), and binding experiments (~ 1 ; Fig. 1, 5, and 6). As an antibody/target protein ratio of ~ 1 is in antibody excess (Fig. 6), we feel that it is very unlikely that aggregation of virus fully explains growth inhibition by MAbs (Fig. 2), thus, factors other than aggregation may well contribute to inhibition of internalization (Fig. 5).

For many viruses, attachment and membrane penetration are multistage processes requiring multiple viral proteins (14). In some cases, initial attachment may result in conformational changes in the virion capsid that are required for subsequent strong attachment and/or penetration. In addition, proteins other than the primary cellular receptor may be involved in internalization of viruses. These considerations lead to alternate hypotheses to explain the effects of $\sigma 3$ MAbs on reovirus internalization. For example, $\sigma 3$ MAbs could prevent conformational alterations in the capsid required for internalization or could inhibit interactions between reovirus capsid proteins (e.g., $\sigma 3$) and proteins on the cell surface required for internalization. This latter mechanism has already been documented for adenovirus (39). We used genetic methods to show that MAb specific for one reovirus capsid protein (e.g., $\sigma 3$) can inhibit the actions of other reovirus capsid proteins (35). Given this fact, binding of MAbs to $\sigma 3$ could inhibit processes involving proteins such as $\sigma 1$, $\lambda 2$, or $\mu 1$ which are required for internalization.

Inhibition of proteolytic uncoating of virus by MAbs. The fact that we could quantitatively remove bound T3D- $\sigma 3$ -10G10 complexes from the cell surface by using proteases allowed us to assess the effects of $\sigma 3$ -10G10 on internalization and subsequent proteolytic uncoating of the virion independently (Fig. 5B and 8, steps B and C). Using this approach, we showed that $\sigma 3$ -10G10 inhibits intracellular proteolytic uncoating of reovirus which is internalized in the presence of the MAb (Fig. 5 and 8, step C). The identity of the intracellular protease(s) responsible for uncoating reovirus is not known, and therefore we could not directly assess the effects of MAbs on uncoating of the virus by a specific intracellular protease. However, chymotrypsin uncoats the virus in a manner similar or identical to cellular uncoating and therefore provided an *in vitro* model for assessing the effects of MAbs on proteolysis of capsid proteins (introduction). Using this fact, we demonstrated that $\sigma 3$ -10G10 directly inhibits proteolytic uncoating of reovirus. Taken together, these experiments suggest that $\sigma 3$ -10G10 likely acts by a combination of inhibition of internalization (Fig. 8, step B) and direct inhibition of the action of intracellular protease(s) responsible for virion uncoating (Fig. 8, step C). Our experiments do not exclude the formal possibility that virion-MAb complexes are shunted to an intracellular site at which proteolysis cannot efficiently occur. We feel that this is less likely, given the demonstration that MAbs can directly inhibit proteolysis of the virion.

Our studies with chymotrypsin also showed that $\sigma 3$ -10C1 and $\sigma 3$ -10G10 inhibit proteolysis of $\sigma 3$ and subsequent pro-

cessing of $\mu 1C$ to δ by different mechanisms. $\sigma 3$ -10G10 appears to inhibit degradation of $\sigma 3$ but allows some processing of $\mu 1C$ to δ (Fig. 7A and B). In contrast, $\sigma 3$ -10C1 is relatively ineffective at preventing proteolysis of $\sigma 3$ but very effective at inhibiting proteolytic cleavage of $\mu 1C$ to δ (Fig. 7A and B). Protection of $\mu 1C$ by $\sigma 3$ -10C1 is associated with preservation of two fragments of $\sigma 3$ (Fig. 7B). This shows that proteolytic processing of $\sigma 3$ is a multistage process and that the step blocked by $\sigma 3$ -10C1 is critical for subsequent processing of $\mu 1C$ to δ . $\sigma 3$ -10G10 and $\sigma 3$ -10C1 recognize distinct but closely related epitopes and cross-compete for binding to virus (35). Thus our results indicate that MAbs with different fine specificities can target multiple steps in the sequential proteolytic uncoating of reovirus. It is intriguing that the $\sigma 3$ capsid protein appears to have two domains in cryoelectron micrographs of the virus (8) and that $\sigma 3$ -10C1 protects two fragments of $\sigma 3$. This suggests a model in which cleavage initially occurs at the junction between the domains and that a conformational change in $\sigma 3$ required for further proteolysis and/or release from the virion is blocked by MAb $\sigma 3$ -10C1. $\sigma 3$ -10C1 could inhibit cleavage of $\mu 1C$ and subsequent conformational changes in the capsid by preventing further cleavage or release of $\sigma 3$. This model can be tested by assessing whether $\sigma 3$ -10C1 MAb and $\sigma 3$ remain associated with the virion after chymotrypsin digestion.

The capacity of antibodies to inhibit proteolytic uncoating of virus may be important *in vivo* as well as *in vitro*. When reovirus enters the intestine by the oral route, proteolytic uncoating of the virion occurs in the intestinal lumen (3). Inhibition of luminal uncoating of reovirus by protease inhibitors limits viral growth in intestines (2). Proteolytic uncoating of reovirus to ISVPs is required for reovirus binding to M cells, which are the entry port for reovirus into Peyer's patches (10a). Antibody can block the luminal proteolysis of reovirions to ISVPs and subsequent binding to M cells (10a). Thus, during natural infection, secretory immunoglobulin A antibodies might inhibit entry of reovirus into the Peyer's patch by inhibiting proteolytic uncoating of virus in the intestinal lumen.

Implications for other viral systems. Our results provide clear evidence of antibody-mediated inhibition of intracellular proteolytic uncoating of an encapsidated virus and thus demonstrate a novel mechanism of antibody action. MAbs which effectively inhibited postbinding steps in reoviral replication were relatively ineffective in producing plaque reduction neutralization. However, there was a good correlation between inhibition of postbinding steps in viral replication and production of small plaques in neutralization assays. Studies in a number of other viral systems indicate that the neutralizing capacity of antibodies does not always correlate with their capacity to inhibit binding to target cell receptors (1, 9, 11, 22). In addition, neutralizing antibodies may remain fully effective even when added after virus attachment to target cells (36), and many nonneutralizing MAbs are protective *in vivo* (reviewed in references 32 and 34). These results show that dependence on simple plaque reduction neutralization may result in lack of attention to important and physiologically relevant mechanisms of antibody action.

A number of postbinding steps at which antibodies can act in other viral systems have been identified. For example, certain influenza virus antibodies inhibit virion internalization (1; reviewed in reference 22). Antibody-mediated inhibition at steps following internalization has also been described. Poliovirus-neutralizing antibody may cross-link virion pentamers and inhibit subsequent viral uncoating without affecting either cell attachment or internalization of virus (9, 10, 15). Antibody inhibits fusion of West Nile virus with intracellular mem-

branes, a step dependent on acidification of intracellular vesicles (12). The intracellular site of action of these West Nile virus antibodies appears to be similar to that of reovirus antibodies that block intracellular proteolytic uncoating, since both of these processes are thought to be endosomal events. Thus our own studies and related work in a number of other viral systems are all consistent with the idea that a prominent and important aspect of antibody action is inhibition of postbinding and intracellular steps in viral replication.

Implications for vaccine and antiviral drug design. A critical issue in the design of subunit vaccines is the selection of appropriate target epitopes. Target epitope selection is complicated by the fact that target epitopes may undergo mutation as a result of selective pressure induced by antibody-based immunity. In this paper we demonstrate that antibodies effectively inhibit critical intracellular steps in viral replication that occur subsequent to virus-cell recognition. Epitopes on the outer surface of viruses which interact with intracellular host cell components may be less likely to undergo mutation as a result of antibody-mediated selective pressure than are viral cell-recognition proteins; they may therefore be excellent targets for vaccine or antiviral drug design. For example, nonreceptor structures involved in viral penetration or structures required for interaction with host cell proteases might provide suitable targets for antiviral immunotherapy. Careful analysis of the effects of antibodies on postbinding steps in the life cycle of a virus may therefore provide a variety of additional and novel candidate target proteins and strategies for both vaccine and antiviral therapy. Our demonstration that antibody can act synergistically with a drug (ammonium chloride) to inhibit the same intracellular processes (Fig. 3) suggests that combining antibody-based and drug-based therapies may have unique advantages.

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