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Preliminary Characterization of an Epitope Involved in Neutralization and Cell Attachment That Is Located on the Major Bovine Rotavirus Glycoprotein

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The 38,200-molecular weight (unreduced)/41,900-molecular-weight (reduced) glycoprotein of bovine rotavirus, isolate C486, was identified as the major neutralizing antigen. This glycoprotein as well as the corresponding glycoprotein of another bovine rotavirus serotype also specifically attached to cell monolayers under normal conditions for virus adsorption in vitro. Further support for this glycoprotein being directly responsible for virus attachment to cells was that (i) infectious virus of both serotypes could compete with the C486 glycoprotein for cell surface receptors, and (ii) neutralizing monospecific antiserum and neutralizing monoclonal antibodies directed toward the glycoprotein could block this virus-cell interaction. Preliminary epitope mapping of the glycoprotein with monoclonal antibodies further localized the neutralization-adsorption domain to a peptide with an approximate molecular weight of 14,000. The effect of two protein modifications, glycosylation and disulfide bridging, on the reactivity of this peptide with antibodies and cell surface receptors was investigated. It was demonstrated that, whereas glycosylation did not appear to affect these reactivities, disulfide bridging seemed to be essential.

Rotaviruses cause important gastrointestinal disorders in animals and humans; however, to date no effective vaccine has been produced. Several approaches have been used for virus vaccine production with recent emphasis on synthetic oligopeptide and recombinant vaccines (1, 19). Vaccines of these types are inherently safer and more stable than the conventional modified live vaccines. However, before such approaches are feasible for a rotavirus vaccine a thorough understanding of the protective antigens is required.

The proteins of infectious rotaviruses exist in a double-shelled arrangement (16). The two outer shell proteins are of particular interest since they play a critical role in important biological properties exhibited by the virus. Specifically, the minor outer shell protein (unreduced/reduced, 82,000 molecular weight [82K]/84K) is responsible for protease-enhanced infectivity (12, 17) and demonstrates hemagglutinating ability (17). The other, more abundant protein (38.2K/41.9K) has been identified as the major neutralizing antigen for U.K. calf, rhesus monkey, and simian rotaviruses (7, 11, 13, 22). Preliminary characterization of this protein identified N-glycosidically linked carbohydrate moieties that appear to stabilize the virus particle (23, 25). In addition, a study of several simian (SA11) (11) and bovine (isolate C486) (24) rotavirus plaque isolates demonstrated heterogeneity in this glycoprotein.

This manuscript presents data on the identification and characterization of the major bovine rotavirus-neutralizing antigen of strain C486. Specific experiments were carried out to elucidate the functional role of this protein in the virion and a possible mechanism by which monoclonal antibody-mediated neutralization occurs. A further attempt was made to characterize a biologically functional domain located on this glycoprotein and investigate the extent to which it was conserved between two different bovine rotavirus serotypes.

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MATERIALS AND METHODS

Virus and cells. Bovine rotavirus isolates C486 and 2352 were cultured from the feces of diarrheic calves by a method described previously (2). This isolate was propagated in African monkey kidney cells (MA-104) in the presence of 10 μg of trypsin (Difco Laboratories, Detroit, Mich.) per ml (2).

MA-104 cells were propagated in Eagle minimal essential media (MEM) supplemented with 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.). To culture virus, confluent MA-104 cells were washed once with MEM and then maintained in the absence of fetal bovine serum for the duration of the infection.

Radiolabeling of virus. The procedure for radiolabeling proteins was to infect cells with virus and then replace the virus inoculum with methionine-free MEM. After a 3-h incubation at 37°C, 25 to 50 μCi of L-[^{35}S]methionine (Amersham, Oakville, Ontario) per ml was added to the overlay.

Preparation of different samples for polyacrylamide gel analysis. When purified virus was desired, infected cell supernatants were harvested 24 h postinfection. After removal of cellular debris by low-speed centrifugation, virus was concentrated by pelleting through a 40% sucrose cushion containing 10 mM $CaCl_2$. The resulting pellet was then layered onto 11.5 ml of CsCl solution (analytical grade, density 1.3688 g/ml; Sigma Chemical Co., St. Louis, Mo.) containing 1 mM $CaCl_2$ and centrifuged at 38,000 rpm in an SW41 rotor (Beckman model L5-65) for 17 h at 15°C. The complete double-shelled particle banded at a density of 1.3692 g/ml.

Fractionation of virus proteins was accomplished by suspending purified virus in Laemmli sample buffer (20) and then electrophoresing in a polyacrylamide slab gel system (20). When reduced proteins were desired, Laemmli sample buffer containing 1, 5, or 15% β-mercaptoethanol was employed. Unreduced proteins were obtained when β-mercaptoethanol was omitted from the sample buffer.

Analysis of proteins from infected cell lysates was employed in situations where the unglycosylated counterpart of the glycoprotein was required. To produce the unglycosylated protein, virus-infected cells were treated with 1 μg of tunicamycin (Sigma) per ml for the duration of the infection. Infected cells were harvested 9 h postinfection by adding Laemmli sample buffer without β-mercaptoethanol. Tunicamycin-treated and untreated radiolabeled infected cell lysates were then fractionated on polyacrylamide gels. After electrophoresis, wet gels were exposed to film (3M; Picker International, Saskatoon, Saskatchewan), and the proteins under consideration were located in and excised from gels. These individual proteins were then electrophoresed for further testing with antibodies.

Adsorption studies. To prepare infected cell lysates for adsorption experiments, radiolabeling was performed as described above, except that infected cells were harvested 8 h postinfection. The virus used for adsorption studies was a subclone of isolate C486 and isolate 2352 (24) so as to eliminate ambiguity when evaluating the results.

Radiolabeled infected cells (5×10^6) were harvested and washed twice with MEM before freezing at −70°C. Once thawed, infected cells were lysed with 400 μl of RSB (0.01 M NaCl, 0.01 M Tris-hydrochloride [pH 7.2], 1.5 mM $MgCl_2$) containing 0.2 mM phenylmethylsulfonyl fluoride and homogenized briefly at 4°C. Nuclei were removed by low-speed centrifugation (2,500 × *g* for 10 min), and then an equal volume of 2× MEM containing 1% fetal bovine serum was added to the supernatant. Lysates were then centrifuged at 45,000 rpm in an SW50.1 rotor (Beckman model L5-65). Adsorption studies were carried out by applying 400 μl of lysate onto a 60- by 15-mm well of MA-104 cells. Incubation proceeded for 1.5 h at 4°C with intermittent rocking. Virus competition and antibody blocking studies were performed by first incubating the lysate with either infectious virus or antibody for 1 h at 37°C before application to cell monolayers.

After adsorption, cell monolayers were washed with cold saline and processed. For identification of adsorbed proteins, 100 μl of Laemmli sample buffer containing 1% β-mercaptoethanol was added per well, and the proteins were fractionated on polyacrylamide slab gels. To determine the amount of radioactivity adsorbed to cells, 1 ml of 1% Triton X-100 was added and then transferred to scintillation vials containing 10 ml of aqueous counting solution (Amersham).

Purification of virus outer capsid polypeptides. Double-shelled virus particles were purified as described above. To purify the outer capsid polypeptides, the double-shelled virus-containing band from a CsCl gradient was first dialyzed overnight against 100 mM Tris-hydrochloride (pH 7.4). After dialysis, the virus was treated with 10 mM EDTA for 30 min at 37°C and then layered onto a preformed 20 to 45% (wt/wt) CsCl gradient and centrifuged at 35,000 rpm for 4 h at 10°C in an SW50.1 rotor. The top 250-μl fraction of the gradient consistently contained the outer capsid polypeptides.

Peptide mapping (Cleveland digest). The protocol for peptide mapping of individual rotavirus polypeptides was described previously (25). After localizing and excising individual ^{35}S-labeled polypeptides from a 10% preparative gel, the gel slices were processed by the procedure of Cleveland et al. (8). The protein in each gel slice was then digested with various enzymes and by chemical cleavage by using cyanogen bromide (Sigma), and the digests were electrophoresed through a 5% polyacrylamide stacking gel–15% resolving gel system. The resulting Cleveland digest was electroblotted to 0.45-μm nitrocellulose paper as described below, and the nitrocellulose strips were then exposed to 3M film overnight at room temperature to visualize the transferred peptide pattern.

Fractionation and isolation of individual virus polypeptides. Complete virus particles were suspended in Laemmli sample buffer and electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide slab gel. When denatured polypeptides were desired, Laemmli sample buffer containing 5% β-mercaptoethanol was employed, and samples were boiled for 5 min. "Undenatured" polypeptides were obtained under conditions where β-mercaptoethanol was omitted from sample preparations.

Individual polypeptides were localized after preparative polyacrylamide gel electrophoresis by staining side strips of the gel with Coomassie brilliant blue R250 (Bio-Rad Laboratories, Richmond, Calif.) and then destaining with 7% acetic acid. Once localized, the corresponding unstained polypeptide bands were excised and placed in 5-ml plastic pipettes. The pipettes were then filled with 0.1× Laemmli running buffer (20), plugged at either end with glass wool, and fitted at the tip with a small dialysis bag containing approximately 400 μl of the above buffer. Electroelution of polypeptides from gel slices into dialysis bags was accomplished after 12 h at a constant current of 2 mA per pipette and employing 0.5× Laemmli running buffer (20). After electroelution, the current was reversed for 2 min to remove adhering polypeptides from the wall of the dialysis bag. Further concentration of polypeptides was accomplished by precipitation in 3 volumes of cold, 95% ethanol, overnight at −20°C. After the precipitate was collected by centrifugation, the pellet was lyophilized and suspended in the desired buffer.

Production of monospecific antisera to individual virus polypeptides. Individual polypeptides from purified virus preparations were fractionated, localized, and isolated from polyacrylamide gels as described above. Virus-specific polypeptides of 38.2K/41.9K, 45K, and 82K/84K were each administered to two rabbits in either of two preparative forms. (i) The acrylamide gel slices containing the appropriate polypeptide were extruded through a 18-gauge and then a 21-gauge hypodermic needle. The broken gel pieces were then homogenized in 2 ml of 0.14 M saline in a tissue homogenizer and sonicated for 30 s (Biosonic sonified disrupter, model W1400) at a setting of 4. (ii) The polypeptides were electroeluted from the gel slices by the method described above.

The first injection of each protein preparation was given in complete Freund adjuvant (GIBCO Laboratories, Burlington, Ontario) and was followed 3 weeks later by a second injection in incomplete Freund adjuvant. Animals were then bled approximately 2 weeks later to monitor the monospecificity and titer of the antisera. Due to low titers, two to three additional booster injections were given at 3-week intervals, after which time the rabbits were bled, and the sera (heat inactivated for 30 min, 56°C) were stored at −70°C.

Production of hybridoma cell lines. (i) Immunization. BALB/c mice were immunized with two different preparations of bovine rotavirus isolate C486. The first inoculum consisted of purified complete rotavirus particles, and the second preparation consisted of purified outer capsid polypeptides prepared as described previously. Two mice were immunized intraperitoneally with each of the two preparations (mixed with an equal volume of complete Freund adjuvant). After 2 weeks, virus was again administered

intraperitoneally with an equal volume of incomplete Freund adjuvant. The mice were finally boosted by a tail vein injection with the appropriate virus preparation. Three days later the mice were sacrificed, and the spleens were removed for fusions.

(ii) Fusion of spleen cells with NS-1 cells. The procedure followed for fusion of spleen cells with NS-1 cells was essentially that of Greenberg et al. (13). After the fusion, wells with visible colonies were tested by enzyme-linked immunosorbent assay (ELISA) against whole virus and individual polypeptides. Selected hybridomas were subcloned by limiting dilution with a macrophage feeder layer and grown to yield a 2- to 5-ml suspension. To amplify some monoclonal antibodies, hybridoma cells were injected intraperitoneally into pristane (Aldrich Chemical Co., Milwaukee, Wis.)-primed BALB/c mice at a concentration of 5×10^5 cells per mouse. Ascitic fluids were collected 1 to 2 weeks later, clarified by low-speed centrifugation, and stored at −20°C.

Plaque reduction assay. Neutralization of bovine rotavirus isolate C486 by rabbit antisera and ascitic fluids was determined by a standard 50% plaque reduction assay. Virus dilutions representing 300 PFU were mixed 1:1 with various dilutions of antibody and incubated for 1 h at 37°C. The antibody concentration in ascitic fluids and antisera was standardized based on ELISA titers. Virus adsorption to MA-104 monolayers was allowed to proceed at 37°C for 2 h before the virus inoculum was removed; the cells were then washed with MEM and overlaid with 1.6% Bacto-agar (Difco) diluted in MEM and supplemented with 5 μg of pancreatin per ml, 0.7% of a 1:1,000 neutral red stock solution, and 0.1% DEAE-dextran. Plaques appeared after 4 to 5 days of incubation at 37°C.

ELISA procedure. The ELISA was carried out by following a previously described procedure (27). Briefly, 96-well microtiter plates (Immulon 2; Dynatech Laboratories Inc., Alexandria, Va.) were incubated at 4°C overnight with an excess of protein (0.50 μg per well). If the antigen was in the form of individual polypeptides, the diluent was 10× carbonate-bicarbonate buffer (pH 9.6). When complete undisrupted virus particles were used as a substrate, the diluent was 1× carbonate-bicarbonate buffer (pH 9.6). The volume of substrate per well was 100 μl.

After incubation of plates with substrate, excess protein was removed by extensive washing in distilled water. The substrate was then overlaid with 75 μl of either rabbit antiserum, monoclonal culture supernatant, or ascitic fluid per well in an undiluted form or diluted in 0.01 M phosphate-buffered saline containing 0.5% fetal bovine serum. Incubation of antigen with antibody was carried out for 1 h at room temperature, after which time excess antibody was removed by washing in distilled water.

Seventy-five microliters of a 1:2,000 dilution of goat anti-rabbit immunoglobulin G (IgG) horseradish peroxidase conjugate (Boehringer Mannheim Biochemical, Calgary, Alberta) or a 1:1,000 dilution of rabbit anti-mouse IgG horseradish peroxidase conjugate (Jackson Immuno Research Labs, Avondale, Pa.) was then added per well, and incubation with the conjugate proceeded at room temperature for an additional hour.

After extensive washing to remove excess conjugate, the bound conjugate was reacted with 75 μl of chromagen and enzyme substrate (recrystallized 5-amino salicylic acid, 1 mg/ml in 0.01 M phosphate buffer, pH 5.95 ± 0.05; Aldrich Chemical Co.) per well, to which 0.005% hydrogen peroxidase was added immediately before use. The reaction was allowed to proceed for 30 min at room temperature before the adsorbance (450 nm) of each well was determined with a micro-ELISA reader (Dynatech).

Western blotting of rotavirus polypeptides and reaction of polypeptides transferred to nitrocellulose with antibody. Virus-specific polypeptides fractionated on 10% polyacrylamide gels and proteolytic digests of these polypeptides fractionated on Cleveland gels (8) were transferred, via electroblotting to nitrocellulose paper (0.45 μm; Schleicher & Schuell Co., Keene, N.H.). The conditions for transfer were as follows: 12 h at 4°C in 25 mM Tris-hydrochloride (pH 8.3)–190 mM glycine–20% methanol at 8.75 V/cm. After transfer, either the nitrocellulose strips were exposed to film, if the polypeptides were radiolabeled, or a protein of the nitrocellulose strips was stained, if unlabeled samples were used, to determine the efficiency of transfer.

The procedure for reacting monoclonal antibodies or rabbit antisera with polypeptides transferred to nitrocellulose was essentially the same as that described by Braun et al. (6). Each 11- by 1.0-cm nitrocellulose strip was incubated with 3 ml of phosphate-buffered saline containing 3% bovine serum albumin (fraction V, Sigma) and either 5 μl of horseradish peroxidase-coupled goat anti-rabbit IgG or horseradish peroxidase-coupled rabbit anti-mouse IgG. Subsequent development of the strips with 0.02% *ortho*-dianisidine dihydrochloride substrate proceeded for 18 h at room temperature.

RESULTS

Identification of the major neutralizing antigen of bovine rotavirus isolate C486. To identify the major neutralizing antigen of bovine rotavirus (isolate C486), monospecific antisera were produced against the two outer shell proteins (ca. 82K/84K and 38.2K/41.9K) and to the major inner shell protein (ca. 45K). Rabbits were immunized with individual proteins both in their reduced (84K, 41.9K) and unreduced (82K, 38.2K) forms (1:1) as described above. The monospecificity of the antisera was tested via ELISA and immunoblot ELISA with whole virus and the reduced and unreduced forms of the antigens (Table 1 and Fig. 1).

Serum neutralization tests were carried out with monospecific antisera raised against the three major rotavirus proteins. Four of the monoclonal antibodies and monospecific antisera against the 38.2K/41.9K protein neutralized virus most effectively (Table 1). Antiserum to the 82K/84K protein also neutralized virus infectivity, but at a much lower dilution. These results are in agreement with previous studies designating the rotavirus major outer shell glycoprotein as the major neutralizing antigen.

Selection of virus-neutralizing monoclonal antibodies specific for the major glycoprotein. Monoclonal antibodies to the rotavirus proteins and in particular to the major glycoprotein were produced using two immunization regimes, as outlined above. Regardless of whether mice were immunized with a whole virus preparation or a purified outer capsid preparation, the percentage of reactive polyclonal antibodies was almost identical (data not shown). However, the percentage of polyclonal antibodies that were predominantly reactive to the glycoprotein as compared with the other viral proteins (i.e., gave the highest ELISA reading against the purified glycoprotein) was greater when hybridomas producing monoclonal antibodies were derived from mice immunized with a purified, outer shell preparation. Since the 45K inner shell protein is the most abundant in the virus, it was not suprising to find that many of the monoclonal antibodies derived from mice immunized with a whole virus preparation are predom-

TABLE 1. Characterization of monospecific antisera and monoclonal antibodies

Antibody type	Reaction[a] with:							Neutralizing titer[e]
	Whole virus[b] (double shelled)	Unreduced[c]			Reduced[d]			
		41.9K (38.2K)	45K	84K (82K)	41.9K	45K	84K	
Polyclonal								
Anti-38.2/41.9K	+	+	+	−	+	+	−	200,000
Anti-45K	+	−	+	−	−	+	−	500
Anti-82K/84K	+	−	−	+	−	−	+	5,000
Monoclonal								
4B5-5	+	+	−	−	−	−	−	10,000
11D10-4	+	+	−	−	−	−	−	10,000
11D12-6	+	+	−	−	−	−	−	25,000
10D2-7	+	+	−	−	−	−	−	100,000
10D2-2	+	+	−	−	−	−	−	500
11D10-1	+	−	−	+	−	−	−	10,000

[a] +, Reactive; −, nonreactive.
[b] Reaction with whole virus determined by ELISA.
[c] Reaction with proteins determined by ELISA and immunoblot ELISA. The apparent molecular weights of the unreduced protein are within parenthesis.
[d] Reaction with proteins treated with β-mercaptoethanol as determined by ELISA and immunoblot ELISA.
[e] Neutralizing titer determined by the reciprocal of antibody dilution necessary to produce a 50% reduction in plaque number. The antibody concentration in ascitic fluids and antisera was standardized based on ELISA titers.

inantly against this protein. To succeed in isolating as many strong anti-38.2K/41.9K glycoprotein monoclonal antibodies as possible, only hybridomas secreting monoclonal antibodies that were predominantly against this glycoprotein were chosen for further subcloning and characterization.

After subcloning, five anti-38.2K/41.9K glycoprotein monoclonal antibodies were selected on the basis of their ability to react with this protein species blotted to nitrocellulose (Fig. 1). The reactivity of monoclonal antibodies with the reduced rotavirus protein blotted to nitrocellulose was somewhat unexpected, especially since they did not react with reduced 41.9K protein in ELISA. This discrepancy, however, was due to the presence of some 38.2K/41.9K in the whole virus polypeptide profile, even in the presence of 1% β-mercaptoethanol (data not shown).

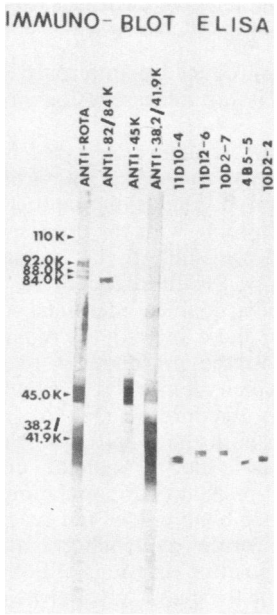

FIG. 1. Reaction of monospecific polyclonal antisera and monoclonal antibodies with the polypeptide profile of double-shelled bovine rotavirus (isolate C486) transferred to nitrocellulose paper.

Mechanism of bovine rotavirus neutralization mediated by antibodies to the major 41.9K glycoprotein. The specific mechanism whereby polyclonal and monoclonal antibodies to the major glycoprotein neutralize bovine rotavirus infectivity was elucidated by determining the function of this protein in the virus particle. The outer shell location of this protein suggested that it might be involved in the attachment to cells in vitro.

Only the major glycoprotein adhered to cells when a radiolabeled infected cell lysate was reacted with a cell monolayer under conditions normally employed for virus adsorption in vitro (Fig. 2). Two observations made from this experiment are of particular interest. First, it is noteworthy that after adsorption of the lysate and before washing the cell monolayers, it appeared that only virus-specified proteins adhered to the monolayer. Subsequent washes, however, removed all of the viral proteins except for the major glycoprotein, as demonstrated by the lack of diminution in intensity after the first saline wash (Fig. 2). Second, despite the fact that adsorption samples for gel analysis were prepared in 1% β-mercaptoethanol, it appears that the unreduced subpopulation of this glycoprotein, which migrates at 38.2K, preferentially adsorbed to cell monolayers (Fig. 2).

Further support implicating the major glycoprotein as the cell attachment protein of the virus was the ability of infectious C486 virus to prevent binding of this glycoprotein to cells in a competitive manner (Fig. 3). Adsorption of isolate C486 virus was also inhibited by polyclonal, monospecific anti-38.2K/41.9K serum and anti-38.2K monoclonal antibodies (Table 2). Although antiserum to the 82K/84K protein also inhibited virus attachment to cells, it was not as dramatic as that exhibited by anti-38.2K/41.9K serum. Anti-82K monoclonal antibodies (11D10-1) and nonneutralizing anti-38.2K monoclonal antibodies did not inhibit this function.

Effect of glycosylation on the reactivity of the bovine rotavirus major glycoprotein. Since the major glycoprotein (38.2K/41.9K) is involved in cellular attachment, the role of

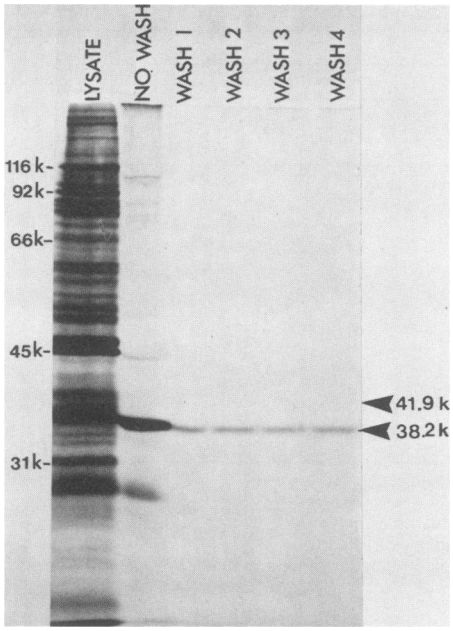

FIG. 2. Identification of the rotavirus cell attachment protein. The adsorption experiment was carried out as described in the text. The samples for analyses on polyacrylamide gels were prepared in 50 μl of Laemmli sample buffer containing 1% β-mercaptoethanol. The cell attachment protein is indicated by the arrow at a molecular weight of 38.2K and was the only band remaining after four washes in saline. The position of the reduced glycoprotein is indicated at a molecular weight of 41.9K. The molecular weight standards are on the left side of the figure.

the *N*-linked carbohydrate moieties in this function was examined. Tunicamycin treatment of bovine rotavirus-infected cells was used to generate the unglycosylated counterpart of the 38.2K glycoprotein. When the unglycosylated (33.1K) protein was reacted with monospecific antiserum to the 38.2K/41.9K protein and monoclonal antibodies from hybridoma 11D12-6, a reaction comparable to that with the glycosylated (38.2K) protein was observed (Fig. 4). Since both the polyclonal and monoclonal antibodies neutralize virus infectivity and block virus attachment to cells in vitro, their indifference to the presence of carbohydrate moieties on the 38.2K protein suggests that glycosylation is not necessary to the reactivity of this protein with these neutralizing antibodies. Furthermore, the unglycosylated and unreduced protein adsorbs to cells in vitro as efficiently as its glycosylated and unreduced counterpart (Fig. 5), implying that glycosylation is not necessary for virus attachment to cells.

TABLE 2. Inhibition of virus adsorption to cells mediated by antiserum and monoclonal antibodies

Antiserum or monoclonal antibody[a]	% Inhibition of adsorption[b]
Rabbit anti-38.2K/41.9K	100
Rabbit anti-82K/84K	39
Rabbit anti-45K	0
10D2-2	0
4B5-5	92
11D10-4	94
11D12-6	97
10D2-7	91
11D10-1	0

[a] The antibody concentration in antisera and from ascitic fluids was standardized based on ELISA titers and then used at a 1:10 dilution.

[b] Percent inhibition was determined as compared with counts adsorbed to cell monolayers when no antibody was added; 0% inhibition was 5×10^4 cpm.

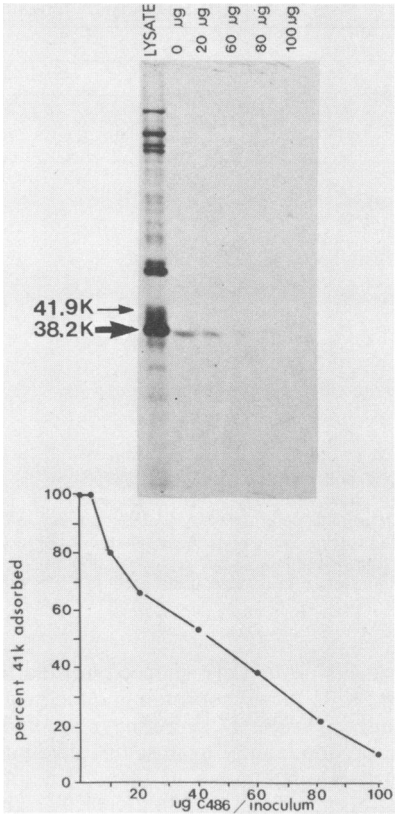

FIG. 3. Competition study between radiolabeled isolate C486-infected cell lysates and unlabeled infectious C486 virus particles. The competing virus was added at concentrations of 0, 5, 10, 20, 40, 60, 80, and 100 μg per inoculum to a standard amount and volume of radiolabeled lysate. The results are depicted on the lower graph as a percentage of the total radioactivity adsorbed to cells when no competing virus was added. One hundred percent was approximately 10^4 cpm. The cell attachment protein is indicated by the arrow. Representative samples were processed for polyacrylamide gel analyses by adding 100 μl of Laemmli sample buffer containing 1% β-mercaptoethanol to cell monolayers in a 60- by 15-mm dish.

Effect of secondary structure, specified by disulfide bridging, on the reactivity of the bovine rotavirus major glycoprotein. A comparison of the β-mercaptoethanol-treated infected cell protein profile with the corresponding untreated profile demonstrates a shift in the mobility of the major glycoprotein from 38.2K (unreduced) to 41.9K (reduced). The appearance of a doublet, identified as a and b in the unreduced position and c and d in the reduced position (Fig. 5), is likely due to the presence of two C486 rotavirus subpopulations, which demonstrate genetic heterogeneity with respect to this glycoprotein (Fig. 6). The residual band (Fig. 5, band e) in both the 5 and 15% β-mercaptoethanol-treated samples is either a cellular contaminant or a β-mercaptoethanol-resistant subpopulation of the glycoprotein. However, since band e does not react with the antirotavirus serum, the former explanation is the most likely.

Contrary to the positive reactions of both the reduced and unreduced forms of the major glycoprotein with monospecific anti-38.2K/41.9K serum and antirotavirus serum, only the unreduced protein appeared capable of reacting with monoclonal antibodies derived from hybridomas 4B5-5,

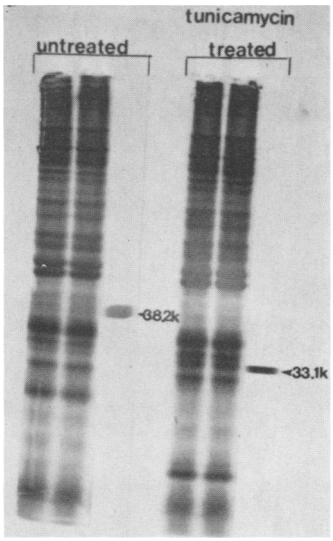

FIG. 4. Reaction of polyclonal and monoclonal antibodies with the glycosylated and unglycosylated forms of the neutralizing antigen. The appropriate proteins were excised from electrophoretic profiles of radiolabeled tunicamycin-treated and untreated infected cell lysates and electrophoresed on a 15% polyacrylamide gel. Proteins shown in the autoradiogram (extreme left lane of each panel) and those transferred to nitrocellulose for reaction with antibodies are in an unreduced form. Polyclonal antibodies against the 38.2K/41.9K protein are designated as anti-41.9K. Monoclonal antibodies were derived from hybridoma 11D12-6.

11D10-4, 11D12-6, and 10D2-7 (Fig. 7, Table 1). Since these monoclonal antibodies specifically neutralized virus infectivity by blocking adsorption (Table 2), one can infer that disulfide bridging is necessary for maintaining the in vitro reactivity of the major glycoprotein with these antibodies and also with cell surface receptors. In fact, adsorption experiments employing radiolabeled, infected cell lysates demonstrated that only the unreduced form of the glycoprotein remained attached to cells after several washes of the monolayer (Fig. 2).

Identification and characterization of the reactive peptide of the neutralizing cell attachment protein. Monoclonal antibodies from hybridoma 11D12-6 were further employed to identify the protein domain specifying the adsorption-neu-

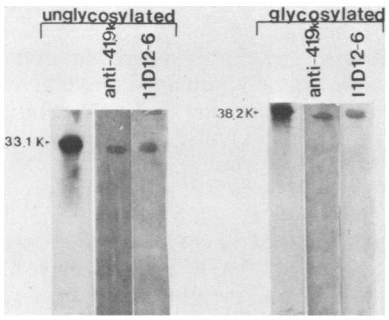

FIG. 5. Effect of glycosylation on the ability of the major glycoprotein to adsorb to cells. Tunicamycin-treated and untreated infected cell lysates were adsorbed to MA-104 cells under normal conditions for virus adsorption. The adsorbed proteins are indicated by the arrows with corresponding molecular weights. The first two lanes in both the treated and untreated panel represent the protein profiles of the radiolabeled infected cell lysates.

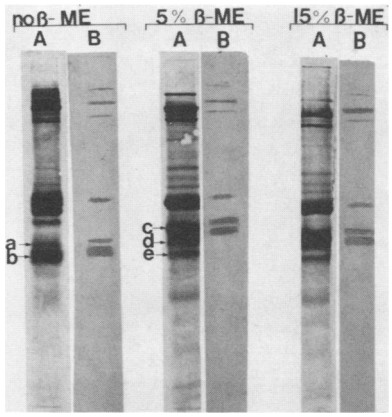

FIG. 6. Reaction of antirotavirus serum with the reduced and unreduced protein profile of bovine rotavirus isolate C486. Protein reduction was accomplished by suspending proteins in Laemmli sample buffer containing 5 or 15% β-mercaptoethanol (β-ME). Lanes A represent autoradiographic images of the protein profiles fractionated on 10% polyacrylamide gels. Lanes B represent the reaction of these protein profiles transferred to nitrocellulose with antiserum. Lowercase letters a and b denote bands in the unreduced position, c and d denote bands in the reduced position, and e denotes a putative cellular protein.

tralization function of the major bovine rotavirus glycoprotein. Specifically, preparations of the glycoprotein were digested with various enzymes or chemically cleaved with cyanogen bromide. The resulting digests were electrophoresed and transferred to nitrocellulose paper. The reaction of these blotted digests with monoclonal antibodies indicated that, regardless of the enzyme or method employed, the smallest immune reactive peptide was ca. 14K (Fig. 8), even though, as illustrated by the corresponding autoradiograms, smaller peptides were generated. Furthermore, since CN-Br cleavage of [^{35}S]methionine-labeled proteins leads to release of ^{35}S, the ^{35}S-labeled 14K peptide must be an incomplete CN-Br cleavage product of glycoprotein 38.2K/41.9K.

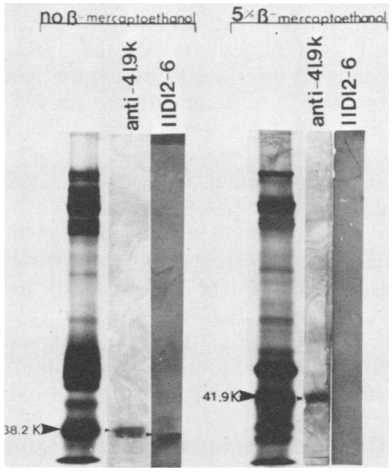

FIG. 7. Reaction of monospecific serum and monoclonal antibodies with the reduced and unreduced profile of bovine rotavirus isolate C486. Protein reduction was accomplished by suspending proteins in Laemmli sample buffer containing 5% β-mercaptoethanol and electrophoresing on a 7.5% polyacrylamide gel. For reaction with antibodies, proteins were transferred to nitrocellulose and processed as described in the text. Monospecific serum against the 38.2K/41.9K protein is designated as anti-41.9K. Monoclonal antibodies were derived from hybridoma 11D12-6.

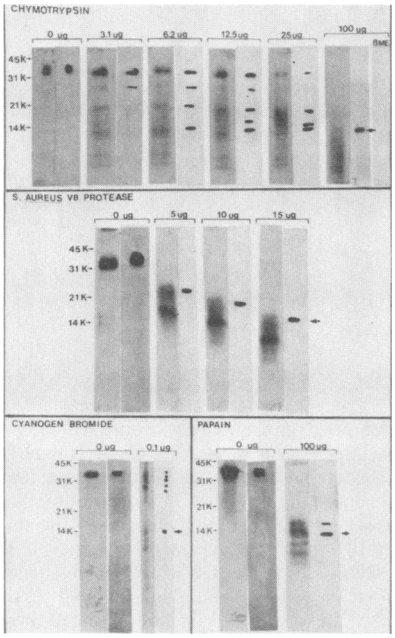

FIG. 8. Reaction of bovine rotavirus glycoprotein digests with monoclonal antibodies from hybridoma 11D12-6. The chemical or enzyme used for digestion is indicated in each panel along with the concentration used. Autoradiograms of the digest are represented in the first of two lanes for each concentration, and the immunoblot reactions are in the second lane. The arrow in the last lane of each panel indicates the 14K peptide. Molecular weight markers are on the left side of each panel. The sensitivity of the 14K peptide to β-mercaptoethanol was tested by excising a piece of nitrocellulose corresponding to this peptide from the identical immunoblot shown in the 100-μg chymotrypsin panel (arrow). This nitrocellulose strip was then boiled in Laemmli sample buffer containing 15% β-mercaptoethanol. The peptide was electrophoresed, blotted to nitrocellulose, and immune reacted with antibodies from hybridoma 11D12-6.

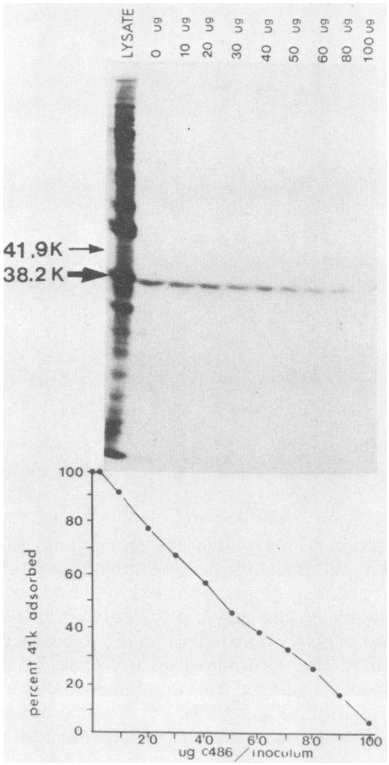

FIG. 9. Competition study between radiolabeled isolate 2352-infected cell lysates and unlabeled infectious C486 virus particles. The competing virus was added at concentrations of 0, 5, 10, 20, 40, 50, 60, 70, 80, 90, and 100 μg per inoculum to a standard amount and volume of radiolabeled lysate. The results are depicted on the lower graph as a percentage of the total radioactivity adsorbed to cells when no competing virus was added. One hundred percent was approximately 5×10^4 cpm. Representative samples were processed for polyacrylamide gel analyses by adding 100 μl of Laemmli sample buffer containing 1% β-mercaptoethanol to cell monolayers in a 60- by 15-mm dish.

The requirememt of disulfide bridges for maintaining the immune reactivity (antigenicity) of the 14K peptide was illustrated by the fact that 15% β-mercaptoethanol treatment eliminated its ability to react with 11D12-6 monoclonal antibodies (Fig. 8, chymotrypsin panel, lane designated β-ME).

Conservation of adsorption-neutralization domain between two bovine rotavirus serotypes. To further investigate whether the cellular receptor that mediates virus adsorption is the same regardless of the serotype of the virus, competition between a radiolabeled isolate 2352 (serotype 3)-infected cell lysate and infectious isolate C486 (serotype 1) virus particles was studied. Figure 9 illustrates that the corresponding major glycoprotein of isolate 2352 preferentially attaches to cells and can effectively compete with C486 virus particles for cellular receptors.

Evidence for the conservation of the adsorption-neutralization domain among at least two bovine rotavirus serotypes was provided by the fact that the major glycoprotein of isolate 2352 reacted with both anti-C486 38.2K/41.9K serum and with monoclonal antibodies (hybridoma 11D12-6) specific for the C486 38.2K major glycoprotein (Fig. 10). In addition, monoclonal antibodies from hybridoma 11D12-6 also neutralized isolate 2352 infectivity as efficiently as isolate C486 infectivity (Table 3), further confirming that this is not a serotype-specific site.

DISCUSSION

Identification of the 38.2K/41.9K bovine rotavirus glycoprotein as the major neutralizing antigen is in agreement with reports of the analogous glycoprotein in various rotavirus isolates eliciting significant neutralizing activity against the homologous virus (7, 11, 13, 22). The neutralizing ability of monospecific antiserum to the minor outer shell protein (92K/84K) was not surprising considering this protein's location on the virion and its role in infectivity (13, 17). However, it is interesting that antiserum to the major inner shell protein (45K) also exhibits a low degree of neutralizing activity. Several explanations can account for this observation. First, the 45K antigen preparation may have been contaminated with the neutralizing antigen, thereby inducing a low level production of neutralizing antibodies. Second, the 45K protein may be somewhat exposed on the virion, thereby enabling a reaction with antibodies that in turn may, via stearic hinderance, block virus attachment to cells. Last, the 45K and 38.2K/41.9K proteins may have some antigenic determinants in common. In this case, even if the 45K protein is not directly involved in neutralization, antibodies to this protein may have neutralizing ability. Support for this possibility is the cross-reactivity of anti-38.2K/41.9K serum with the 45K protein

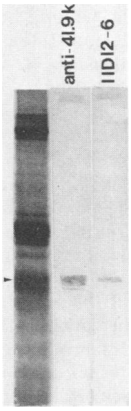

FIG. 10. Immune reactivity of the major glycoprotein of isolate 2352 with antibodies to the corresponding glycoprotein of isolate C486. The left lane shows an autoradiograph of the protein profile of isolate 2352 prepared in Laemmli sample buffer with no β-mercaptoethanol. The reaction of this protein profile, transferred to nitrocellulose, with anti-C486 38.2K/41.9K serum and with monoclonal antibodies from hybridoma 11D12-6 are in the next two lanes.

(Table 1). Specifically, this serum reacts with only four 45K peptides, generated via proteolytic digestion of the 45K protein, suggesting that there are antibody subpopulations in anti-38.2K/41.9K serum that recognize both the 38.2K/41.9K and the 45K protein (26). Unequivocal support for this possibility awaits the identification of monoclonal antibodies that react withboth proteins.

Since the 38.2K/41.9K neutralizing antigen is an abundant outer shell protein responsible for virus attachment to cells in vitro, it is plausible that one mechanism of virus neutralization involves coating of the virus particle with sufficient monospecific polyclonal antibodies to prevent any virus-cell interaction. Another, more precise mechanism was demonstrated by employing monoclonal antibodies whose action was specifically directed to the adsorption-neutralization domain of this protein, thereby blocking virus attachment to cells. Two possibilities exist for the site of monoclonal antibody attachment. Viral adsorption to cells may be blocked by direct binding of these antibodies at or near the putative receptor binding site, or antibodies may bind some distance away from this site, thereby inducing a conformational change that alters the receptor-binding region (14, 15). The fact that conditions that alter the adsorption ability of the protein also affect its antigenic reactivity with these monoclonal antibodies suggests that the antibodies are binding at or near the adsorption-neutralization domain.

Identification of a biologically functional domain was important for two reasons. First, such a domain is likely to be conserved among different rotavirus serotypes, as was suggested by the data presented here; second, only one antibody per site is then required to neutralize virus infectivity. Further studies are being carried out to determine the extent of such conservation. Analyses of nucleotide sequence data of the corresponding genes coding type-specific antigens from different rotaviruses will aid in such studies (5, 10).

TABLE 3. Neutralizing ability of monospecific antiserum and monoclonal antibodies (11D12-6)

Virus	Anti-C486 38.2K/41.9K serum	11D12-6 antibody	Neutralizing titer[a]
Isolate C486	×		200,000
(Serotype 1)		×	25,000
Isolate 2352	×		200
(Serotype 3)		×	20,000

[a] Neutralizing titer determined by the reciprocal of antibody dilution necessary to produce a 50% reduction in plaque number. The antibody concentration in ascitic fluids and antisera was standardized based on ELISA titers.

Further characterization of the functional domain localized it to a 14K peptide. Difficulties encountered while attempting to decrease the size of this peptide led to a closer evaluation of its reactivity as well as that of its parent protein with respect to two common virus protein modifications, i.e., disulfide bridging and glycosylation. The finding that glycosylation did not play a role in the reactivity of the major glycoprotein with cell surface receptors was not too surprising, since Petrie et al. (23) have reported that a simian rotavirus variant lacking any detectable carbohydrate residues was capable of infecting cells in vitro. In addition, it appears that the lack of sugar residues also does not influence glycoprotein reactivity with neutralizing antibodies (Fig. 4). The almost universal modification of virus-neutralizing antigens by the addition of carbohydrate side chains has provoked numerous hypotheses that sugar residues are important to antigenicity, immunogenicity, secondary structure, and stability of the particular protein and consequently of the virus particle (18, 21). For rotaviruses, there is preliminary evidence that virus stability in vitro may be determined by the presence of carbohydrate moieties on the major outer shell protein (23, 25). However, to date, not enough information has been accumulated to assign any one of the above characteristics as being exclusively specified by sugar residues. It is possible that carbohydrate residues may assume different roles in different virus systems.

The inability of neutralizing monoclonal antibodies to recognize the 14K peptide as well as its parent protein in the reduced form indicates that secondary structure, as specified by disulfide bridges, is necessary for maintaining the antigenicity of the adsorption-neutralization domain. This supports the work of Bastardo et al. (3), which demonstrated that antisera to the reduced outer shell proteins of the virus did not neutralize virus infectivity, whereas antisera produced against the unreduced form of the glycoprotein contained type-specific neutralizing antibodies.

Protein conformation also appears to be necessary for maintaining the reactivity of the major glycoprotein with cell surface receptors. Attachment of only the unreduced form of the glycoprotein to cell monolayers suggests that disulfide bridging must occur shortly after translation, since this appears to be the predominant protein form in infected cell lysate profiles. This observation is supported by the close association of the disulfide interchange enzyme with the rough endoplasmic reticulum (4) and by biochemical and ultrastructural studies demonstrating the involvement of this organelle in the morphogenesis of rotaviruses (23, 25). The inability of 1% β-mercaptoethanol to reduce this protein sufficiently to promote a shift suggests the presence of substantial intramolecular disulfide bridging. Further support for the presence of disulfide bridges in the 14K peptide is the inability of enzymes, which are varied in their cleavage sites and chemical cleavage by cyanogen bromide, to further break down this peptide when disulfide bridging is maintained.

The potential role of disulfide bridges in determining protein immunogenicity has been illustrated by a synthetic peptide mimicking a protein of the hepatitis B antigen.

Specifically, by cyclyzing such a synthetic peptide via a disulfide bridge a neutralizing antibody response could be elicited in mice after a single injection without further linkage of this peptide to a protein carrier (9). Based on this, further studies are under way to test the immunogenicity of the rotavirus 14K peptide and to more precisely map it by employing an array of monoclonal antibodies.

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LITERATURE CITED

1. **Arnon, R., M. Shapira, and C. O. Jacob.** 1983. Synthetic vaccines. J. Immunol. Methods **61**:261–273.
2. **Babiuk, L. A., K. Mohammed, L. Spence, M. Fauvel, and R. Petro.** 1977. Rotavirus isolation and cultivation in the presence of trypsin. J. Clin. Microbiol. **6**:610–617.
3. **Bastardo, J. W., J. L. McKimm, O. Breschkin, S. Sonza, L. D. Mercer, and I. H. Holmes.** 1981. Preparations and characterization of antisera to electrophoretically purified SA11 virus polypeptides. Infect. Immun. **34**:641–647.
4. **Bergman, L. W., and W. M. Kuehl.** 1979. Formation of intermolecular disulphide bonds on nascent immunoglobulin polypeptides. J. Biol. Chem. **254**:5690, 8869.
5. **Both, G. W., J. S. Mattick, and A. R. Bellamy.** 1983. Serotype-specific glycoprotein of simian II rotavirus: coding assignments and gene sequence. Proc. Natl. Acad. Sci. U.S.A. **80**:3091–3095.
6. **Braun, D. K., L. Perpeira, B. Norrild, and B. Roizman.** 1983. Application of denatured, electrophoretically separated and immobilized lysates of herpes simplex virus-infected cells for detection of monoclonal antibodies and for studies of the properties of viral proteins. J. Virol. **46**:103–112.
7. **Bridger, J. C.** 1978. Location of type-specific antigens in calf rotaviruses. J. Clin. Microbiol. **8**:625–628.
8. **Cleveland, D. W., L. G. Fisher, N. W. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in SDS and analysis by gel electrophoresis. J. Biol. Chem. **252**: 1102–1106.
9. **Dreesman, G. R., Y. Sanchez, I. Ionescu-Matiu, J. T. Sparrow, H. R. Six, D. L. Peterson, F. B. Hollinger, and J. L. Melnick.** 1982. Antibody to hepatitis B surface antigen after a single inoculation of uncoupled synthetic HBs Ag peptides. Nature (London) **295**:158–160.
10. **Elleman, T. C., P. A. Hoyne, M. L. Dyall-Smith, I. H. Holmes, and A. A. Azad.** 1983. Nucleotide sequence of the gene encoding the serotype-specific glycoprotein of UK bovine rotavirus. Nucleic Acids Res. **11**:4689–4701.
11. **Estes, M. K., D. Y. Graham, R. F. Ramig, and B. D. Ericson.** 1982. Heterogeneity in the structural glycoprotein (VP7) of simian rotavirus SA11. Virology **122**:8–14.
12. **Flores, J., I. Perez, L. White, M. Perez, A. R. Kalica, R. Marquina, R. G. Wyatt, A. Z. Kapikian, and R. M. Chanock.** 1982. Genetic relatedness among human rotaviruses as determined by RNA hybridization. Infect. Immun. **37**:648–655.
13. **Greenberg, H. B., J. Valdesuso, K. van Wyke, K. Midthun, M. Walsh, V. McAuliffe, R. G. Wyatt, A. R. Kalica, J. Flores, and Y. Hoshino.** 1983. Production and preliminary characterization of monoclonal antibodies directed at two surface proteins of rhesus rotavirus. J. Virol. **47**:267–275.
14. **Heinz, F. X., R. Berger, W. Tuma, and C. Kunz.** 1983. Location of immunodominant antigenic determinants on fragments of the tick-borne encephalitis virus glycoprotein: evidence for two different mechanisms by with antibodies mediate neutralization. Virology **130**:485–501.
15. **Heinz, F. X., C. Mandl, R. Beger, W. Tuma, and C. Kunz.** 1984. Antibody-induced conformational changes result in enhanced avidity to antibodies to different antigenic sites on the tick-borne encephalitis virus glycoprotein. Virology **133**:25–34.
16. **Holmes, I. H., B. J. Ruck, R. F. Bishop, and G. P. Davidson.** 1975. Infantile enteritis viruses: morphogenesis and morphology. J. Virol. **16**:937–943.
17. **Kalica, A. J., J. Flores, and H. B. Greenberg.** 1983. Identification of the rotaviral gene that codes for hemagglutination and protease-enhanced plaque formation. Virology **125**:194–205.
18. **Keil, W., H. Niemann, R. T. Schwartz, and H. D. Klenk.** 1984. Carbohydrates of influenza virus. V. Oligosaccharides attached to individual glycosylation sites of the hemagglutinin of Fowl Plaque Virus. Virology **133**:77–91.
19. **Kleid, D. G., D. Yansura, B. Small, and D. Dowbenko.** 1981. Cloned viral protein vaccine for foot-and-mouth disease: response in cattle and swine. Science **214**:1125–1128.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Leavitt, R., S. Scklesinger, and L. Konnfeld.** 1977. Tunicamycin inhibits glycosylation and multiplication of sindbis and vesicular stomatitis viruses. J. Virol. **21**:375–385.
22. **Matsumo, S., and L. Inouye.** 1983. Purification of an outer capsid glycoprotein of neonatal calf diarrhea and preparation of its antisera. Infect. Immun. **39**:155–158.
23. **Petrie, B. L., M. K. Estes, and D. Y. Graham.** 1983. Effects of tunicamycin on rotavirus morphogenesis and infectivity. J. Virol. **46**:270–274.
24. **Sabara, M., and L. A. Babiuk.** 1983. The significance of rotavirus eletrophoretypic heterogeneity within individual bovine rotavirus isolates, p. 95–106. *In* R. W. Compans and D. H. L. Bishop (ed.), Double-stranded RNA viruses. Elsevier Biomedical, New York.
25. **Sabara, M., L. A. Babiuk, J. Gilchrist, and V. Misra.** 1982. Effect of tunicamycin on rotavirus assembly and infectivity. J. Virol. **43**:1082–1090.
26. **Sabara, M., E. James, J. Gilchrist, L. A. Babiuk, and G. R. Hudson.** 1983. A study of the bovine rotavirus type-specific antigen, p. 28–48. *In* S. D. Acres (ed.), VIDO's Fourth International Symposium on Neonatal Diarrhea, 1983. Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan.
27. **Voller, A., and D. Bidwell.** 1975. A simple method of detecting antibodies to rubella. Br. J. Exp. Pathol. **56**:338–339.