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Purification of an Outer Capsid Glycoprotein of Neonatal Calf Diarrhea Virus and Preparation of Its Antisera

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An outer capsid glycoprotein, VP 7, was purified from a bovine rotavirus, neonatal calf diarrhea virus, by isoelectric focusing in glycerol gradients after disruption of the outer capsid. Its isoelectric point was found to be about 4.5. Guinea pigs were immunized with this VP 7 preparation. The antisera possessed both neutralizing and hemagglutination-inhibiting activities as well as complement-fixing activity, suggesting that VP 7 is a protein involved in hemagglutination and initiation of infection. When these antisera were reacted with a simian rotavirus, their antibody titers were low by hemagglutination-inhibiting and complement-fixing assays, but one serum neutralized the simian rotavirus as efficiently as it did homologous neonatal calf diarrhea virus.

Studies of the immune responses to animal and human rotaviruses have shown that type-specific neutralizing and hemagglutination-inhibiting antibodies develop after either primary infection or immunization with virions (2, 3, 5, 10, 12). Immune electron microscopic studies have suggested that the inner capsid antigens are common group antigens, whereas the outer capsid antigens are type specific (13). We reported previously that the outer capsid of neonatal calf diarrhea virus (NCDV) consists of VP 5, VP 7, and VP 8, and that VP 7 is glycosylated (8). Because VP 7 is the major polypeptide of the outer capsid, it is possible that VP 7 is the protein involved in hemagglutination. If such is the case, VP 7 should induce type-specific hemagglutination-inhibiting and neutralizing antibody. In this study, we purified VP 7 from virions, immunized guinea pigs with VP 7, and studied the reactivity of the antisera with homologous and heterologous rotaviruses.

MATERIALS AND METHODS

Cells and viruses. MA-104 cells (macacus rhesus monkey kidney cells) were grown as monolayer cultures in Eagle minimal essential medium containing 10% newborn calf serum. NCDV (Lincoln strain) and simian agent 11 (SA11) virus were grown in MA-104 cells as described previously (9).

Purification of NCDV virions. NCDV-infected MA-104 cells were frozen and thawed three times. After centrifugation for 30 min at 15,000 × *g*, the supernatant fluid was mixed with 8% polyethylene glycol 6000 and 0.5 M NaCl, kept overnight at 4°C, and centrifuged at 15,000 × *g* for 30 min. The pellet was suspended in phosphate-buffered saline to 2% of the original volume, and cesium chloride (CsCl) was added to a final density of 1.36 g/ml. The sample was centrifuged for 16 h at 35,000 rpm in a Beckman SW50.1 rotor at 4°C. Two bands were formed at buoyant densities of 1.36 and 1.38 g/ml. The virion band at the density of 1.36 g/ml was collected.

Isoelectric focusing in glycerol gradients. The method of Korant and Lonberg-Holm (7) for electrofocusing in small-size columns was employed. The apparatus was made by Sibata Chemical Apparatuses Manufacturing Co., Tokyo, Japan. The lower end of the glass column (length 20 cm, internal diameter 1.0 cm) was stoppered with a dialysis membrane. Glycerol gradients of 40 to 10% (wt/vol) containing 1% ampholyte of pH range 3.5 to 10 (LKB Produkter, Bromma, Sweden) were made in the column to the 17-cm height. Samples containing 1% ampholyte and 6% glycerol, and then 1% ampholyte containing 3% glycerol, were layered on the gradients. The lower reservoir with anode contained 0.15 M phosphoric acid and 40% glycerol, and the upper one contained 0.25 M sodium hydroxide. Electrofocusing was done with constant power of 0.75 W per column for 6 h at 5°C. For fractionation of gradients, the lower end membrane was reinforced with Parafilm (American Can Co., Neenah, Wis.). The gradient was overlaid with water containing phenol red. The bottom membrane was pierced by a needle, the water was pumped onto the top at a constant speed, and fractions of constant volume were collected from the bottom.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Matsuno and Mukoyama (11). Samples for electrophoresis were precipitated with 3 volumes of ethanol at −20°C overnight. The precipitates were dried, dissolved in 6 M urea–1% SDS–2% 2-mercaptoethanol–0.005% bromophenol blue, and boiled for 2 min. Separation gels contained 13% acrylamide and 0.1% bisacrylamide. Electrophoresis was run at 120 V for 4 h, and gels were stained with Coomassie brilliant blue for photographing.

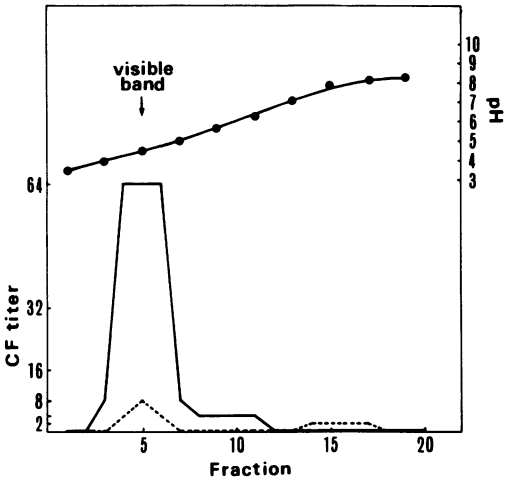

FIG. 1. Isoelectric focusing of solubilized outer capsid proteins. Samples containing VP 7 were electrofocused in 1% ampholyte (pH 3.5 to 10) in 40 to 10% glycerol gradients. ------, CF antigen titers obtained with anti-NCDV virion serum. ——, CF antigen titers with anti-VP 7 serum.

Serological tests. Plaque-reduction neutralization, hemagglutination-inhibition (HI), and complement-fixation (CF) tests were carried out as previously described (9, 10).

Immunoprecipitation. [^{35}S]methionine labeling and purification of virus was carried out as previously described (11). Purified NCDV labeled with [^{35}S]methionine was disrupted with 10 mM EDTA (pH 7.0)–5 M urea at 56°C for 30 min. After overnight dialysis against STE (0.02 M Tris-hydrochloride–0.1 M NaCl–0.001 M EDTA [pH 7.6]) buffer, the samples were centrifuged at 35,000 rpm for 2 h at 10°C in an SW50.1 rotor. The supernatants were diluted with a reaction buffer consisting of 0.01 M Tris-hydrochloride (pH 7.6), 0.14 M NaCl, 0.5% sodium deoxycholate, and 0.5% Nonidet P-40. Anti-NCDV virion and VP 7 sera (final dilution 1:40) were added to the viral protein preparations, and the mixtures were incubated for 1 h at 4°C. Then the samples were mixed with protein A-Sepharose CL-4B gel (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), incubated for 1 h at 4°C, and centrifuged at 15,000 rpm for 5 min in an Eppendorf microcentrifuge. The pellet was washed three times with the reaction buffer and finally suspended in 100 μl of sample buffer and boiled for 2 min. The samples were again centrifuged at 15,000 rpm for 5 min, and the supernatants were applied to SDS-PAGE.

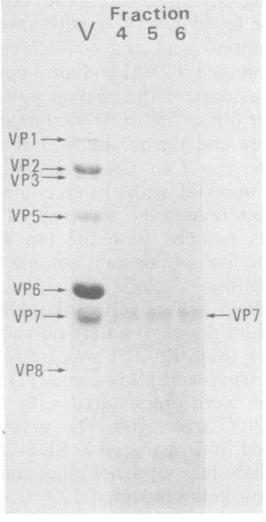

FIG. 2. SDS-polyacrylamide gel electrophoresis of fractions from isoelectric focusing. V: Virion.

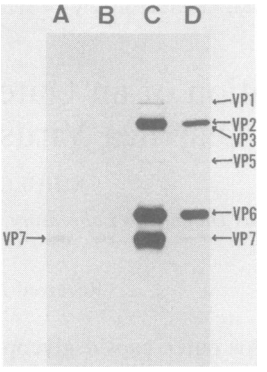

FIG. 3. SDS-polyacrylamide gel electrophoresis of the immunoprecipitates made by anti-NCDV virion and anti-VP 7 sera. Lane A, immunoprecipitates of disrupted virion by anti-VP 7 no. 1 serum; lane B, immunoprecipitates of disrupted virion by anti-VP 7 no. 2 serum; lane C, [^{35}S]methionine-labeled virion; lane D, immunoprecipitates of disrupted virion by antivirion serum.

Inhibition of the adsorption of virion to MA-104 cells by anti-VP 7 sera. A [^{35}S]methionine-labeled NCDV virion preparation in minimal essential medium was divided into 0.1-ml aliquots (1,000 cpm) to which 0.1 ml of anti-VP 7 sera or preimmunization guinea pig serum was added. After 1 h at 37°C, the virion-antisera mixtures were added to monolayers of MA-104 cells growing in six-well plates (diameter 35 mm). After incubation for 2 h at 37°C, the cells were washed three times with cold STE buffer and lysed with STE buffer containing 1% Nonidet P-40. The lysates were then mixed with 10 volumes of 10% trichloroacetic acid, the resultant precipitates were collected on Whatman glass filters, and their radioactivity was counted in a liquid scintillation counter.

RESULTS

Purification of VP 7. Purified NCDVs were treated with 10 mM EDTA at 37°C for 30 min to release the outer capsid proteins (4), which were then separated from the inner capsids by centrifugation in preformed gradients of 20 to 45% (wt/wt) CsCl at 35,000 rpm for 4 h at 10°C in a

TABLE 1. Comparison of hemagglutination inhibition (HI), neutralization (NT), and complement fixation (CF) antibody titers of anti-NCDV VP 7 sera

Serum no.	HI titer against		NT titer against		CF titer[a] against	
	NCDV	SA11	NCDV	SA11	NCDV	SA11
1	1,024	64	9,000	6,600	2,048	256
2	256	8	12,800	30	1,024	128

[a] Results represent reciprocals of the highest antiserum dilution reacting with 4 to 16 CF antigen units.

Spinco SW50.1 rotor. The top fractions were collected. After dialysis against phosphate-buffered saline, the top fractions containing the outer capsid proteins were again centrifuged at 35,000 rpm for 2 h to remove contaminating inner capsids. The supernatant was then treated with 5 M urea at 56°C for 30 min for complete solubilization of protein aggregates. After dialysis against 0.015 M Tris-glycine (pH 7.6), the sample was subjected to electrofocusing as described in Materials and Methods. At fraction no. 5, with an isoelectric point of 4.5, a slightly turbid band was obtained (Fig. 1). By SDS-PAGE, this band was shown to contain only VP 7 (Fig. 2). When this fraction was tested by CF with an antiserum to whole NCDV, the antigen titers were low. However, when an anti-VP 7 antiserum (see below) was used, the titers became higher (Fig. 1).

Preparation of monospecific antisera to VP 7. A 1-ml volume of the purified VP 7 preparation (pooled from fractions no. 4 to 6) was mixed with an equal volume of Freund complete adjuvant and injected into the footpads of two guinea pigs. After 3 weeks, 1.0 ml of the VP 7 preparation without adjuvant was given intraperitoneally to each animal as a booster immunization. The specificity of the antisera produced was then assessed by immunoprecipitation. Sera from both animals precipitated only VP 7 polypeptide (Fig. 3), indicating monospecificity of the antisera. These two antisera were used in the following study.

Reactivity of anti-VP 7 antisera. The reactivity of the antisera was investigated in neutralization, HI, and CF tests, using NCDV and SA11 as antigen. Both antisera possessed neutralization and HI activities against NCDV (Table 1). When they were reacted with SA11 by HI and CF, the antibody titers were lower. However, unexpectedly, no. 1 serum neutralized SA11 as well as it neutralized NCDV.

Next, the ability of anti-VP 7 sera to prevent the adsorption of NCDV virions to MA-104 cells was measured. It was found that the two antisera inhibited the adsorption of NCDV virions (Table 2). This result indicates that VP 7 is one of the cell attachment polypeptides.

TABLE 2. Inhibition of the adsorption of NCDV virion to cells by anti-VP 7 sera

Antiserum	Serum dilution	Adsorbed radioactivity (cpm)	% Inhibition
No. 1	500	122	80
	5,000	501	24
No. 2	500	193	68
	5,000	353	47
Preimmunization guinea pig serum	500	611	0
	5,000	663	0

DISCUSSION

In this study, we purified VP 7 of NCDV in a form which may preserve both antigenicity and immunogenicity. The antisera produced by this preparation were monospecific to VP 7 and had neutralizing as well as hemagglutination-inhibiting activities. VP 7 is probably one of the crucial proteins which maintain the infectivity, hemagglutinating activity, and cell attachment site of NCDV. Recently, Bastardo et al. (1) suggested that the gp34 polypeptide of SA11 was capable of inducing hemagglutination-inhibiting and neutralizing antibodies. And we consider that gp34 of SA11 by Bastardo et al. (1) may correspond to VP 7 of NCDV in our system.

Surprisingly to us, one of the antisera neutralized SA11 to the same extent that it neutralized NCDV, although the HI reaction was type specific (Table 1). VP 7 may have both type-specific and common antigenic sites for neutralization. In this respect, VP 7 seems similar to σ3 polypeptide of reovirus; Hayes et al. (6) reported that rabbit antisera and also monoclonal antibodies to σ3 neutralized reoviruses of homologous and heterologous types but were type specific by HI tests.

The heterotypic neutralizing reactivity of the antisera is intriguing. In 1977, we found that schoolchildren developed high-titered neutralizing antibodies to (non-human) NCDV after acute illnesses of diarrhea and vomiting (7). Further work should be done for more knowledge of rotavirus polypeptides which can elicit heterotypic neutralizing antibodies. It is important not only for understanding the protection mechanism of the host to different serotypes of rota-

virus, but also for seeking strategies in developing effective vaccines.

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