JOURNAL OF VIROLOGY, Aug. 1983, p. 267–275
0022-538X/83/080267-09$02.00/0

Vol. 47, No. 2

Production and Preliminary Characterization of Monoclonal Antibodies Directed at Two Surface Proteins of Rhesus Rotavirus

HARRY B. GREENBERG,* JOSE VALDESUSO, KATHLEEN VAN WYKE, KAREN MIDTHUN, MARGARET WALSH, VINCENT MCAULIFFE, RICHARD G. WYATT, ANTHONY R. KALICA, JORGE FLORES, AND YASUTAKA HOSHINO

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20205

Received 24 January 1983/Accepted 4 April 1983

A series of monoclonal antibodies was isolated which reacted with one of two major surface proteins of rhesus rotavirus. Thirty-six monoclonal antibodies immunoprecipitated the 82-kilodalton outer capsid protein, the product of the fourth gene, the viral hemagglutinin. These monoclonal antibodies exhibited hemagglutination inhibition activity and neutralized rhesus rotavirus to moderate or high titer. Three monoclonal antibodies immunoprecipitated the 38-kilodalton outer capsid glycoprotein, the eighth or ninth gene product. These three monoclonal antibodies neutralized rhesus rotavirus to high titer and also inhibited viral hemagglutination.

Rotaviruses, newly characterized members of the Reoviridae family, are an important cause of infantile diarrhea in many mammalian species, including humans (7, 33). Unlike the mammalian reoviruses, it appears that most mammalian rotavirus strains have a restricted host range. In addition, rotaviruses exhibit a remarkable degree of tissue tropism in vivo, replicating almost exclusively in the villous tip cells of the small intestinal epithelium (30, 33).

As part of a series of studies aimed at examining rotavirus tropism for cell and host, we have isolated a number of monoclonal antibodies directed at two major surface proteins of rhesus rotavirus (RRV). RRV was chosen for study because it grows well in tissue culture, it is serotypically identical to one of the three major human rotavirus serotypes, and we have already determined the gene-coding assignments for several important RRV functions (15, 31). In addition, the availability of an RRV strain propagated only in primary cell culture (29) and its relative attenuation in experimental animals make this virus a promising vaccine candidate (R. Wyatt and E. Bohl, unpublished data). In this study RRV monoclonal antibodies have been used to verify gene-coding assignments for the viral hemagglutinin (gene 4) and neutralizing protein (gene 8 or 9) that were made independently by combined genotypic and phenotypic analysis of viral reassortants (15). In addition, the monoclonal antibodies were used to investigate the antigenic relationships of the viral hemagglutinins and neutralizing proteins of a variety of separate mammalian rotavirus strains. Finally, selected monoclonal antibodies were used to demonstrate that neutralization of rotavirus can be mediated by antibody to the 82-kilodalton (kd) (the viral hemagglutinin) as well as the 38-kd protein.

MATERIALS AND METHODS

Viruses. RRV strain 2 (MMU 18006) was originally obtained from N. Schmidt (29). It was passaged in MA104 cells before use for immunization or immunoassay. Temperature-sensitive (*ts*) mutants of RRV were generated by cultivating the virus in the presence of 5-azacytidine (400 mg/ml) in MA104 cells, using a technique similar to that previously described (10). Bovine rotavirus (UK strain) × RRV reassortants were isolated from MA104 cell cultures coinfected at 34°C with *ts* mutants of both viruses (15). The growth yield of the coinfected cultures was plaqued at 39.5°C. Discrete plaques were picked and plaque purified twice at 39.5°C before amplification for use in solid-phase immunoassays or hemagglutination inhibition (HI) assays. The UK strain was isolated by G. Woode and J. Bridger and was originally provided by T. Flewett (2). It was propagated in MA104 cells. Canine rotavirus strain CU-1 was provided by Y. Hoshino (14). Nebraska calf diarrhea virus, bovine strain, was provided by C. Mebus. OSU porcine rotavirus was provided by E. Bohl and K. Theil. SA-11 rotavirus was provided by H. Malherbe. All rotavirus strains and reassortants were grown in MA104 cells in the presence of trypsin (0.5 μg/ml).

Analysis of the genotype of viral reassortants. The genotype of the UK rotavirus × RRV reassortants was determined as previously described (8, 15). Briefly,

^{32}P-labeled single-stranded mRNAs from both parents were synthesized in vitro by utilizing the endogenous RNA-dependent RNA polymerase present in rotaviral cores (4). Reassortant virion double-stranded RNA was denatured by heating at 100°C for 2 min and then hybridized to ^{32}P-labeled single-stranded RNA transcripts from either parent. The hybridization mixtures were then electrophoresed on polyacrylamide gel and autoradiographed (15).

Immunization. Two female BALB/c mice (952 and 954) were immunized with RRV strain 2, which was partially purified by high-speed pelleting, fluorocarbon extraction, and rate zonal sedimentation through a 20 to 40% sucrose gradient (SW40 rotor; 35,000 rpm for 90 min). The fractions with the peak viral hemagglutination (HA) titer were used for immunization. Both mice were initially immunized intraperitoneally with virus mixed with an equal volume of complete Freund adjuvant. One to 2 months later virus was again administered intraperitoneally with an equal volume of incomplete Freund adjuvant. The mice were again boosted 1 to 2 months later by intravenous inoculation of complete virus in phosphate-buffered saline (PBS). Three days after the intravenous boost the mice were sacrificed and spleens were removed for fusions. At the time of sacrifice both mice had a serum HI titer of >1:640 against RRV.

Fusion of spleen and NS-1 cells. The spleen cells were suspended in Dulbecco minimal essential medium (MEM) with gentamicin and pelleted (500 rpm for 5 min). The resuspended spleen cells were counted by trypan blue exclusion and then mixed with NS-1 myeloma cells at a ratio of 10:1. The spleen cell–NS-1 cell mixture was again centrifuged (1,000 rpm for 5 min), and the supernatant was removed. The cell pellet was shaken gently, and 1 ml of 50% polyethylene glycol 1000 in Dulbecco MEM was added over a 1-min period. Over a 5-min period, 25 ml of Dulbecco MEM was gradually added to the reaction, and the cells again were centrifuged at 1,000 rpm for 5 min. The supernatant was decanted, and the pellet was suspended in HAT (hypoxanthine [13.6 mg/liter]–aminopterin [0.19 mg/liter]–thymidine [15.5 mg/liter]) medium (15% fetal calf serum) and seeded in 96-well Costar plates at 3.5×10^6 spleen cells per ml (19). Plates were maintained at 37°C in 10% CO_2. Fresh HAT medium was added to wells when evaporation occurred. Wells with visible hybridoma colonies were tested by radioimmunoassay (RIA) and HI assays when the medium became acid or the monoclonal cells covered greater than one-fifth of the well bottom or both. Hybridomas of interest were cloned once or twice by limiting dilution, using a thymocyte feeder layer, and then grown to yield a 20- to 40-ml suspension that was used for subsequent testing. Some of the monoclonal antibodies were amplified by injecting the hybridoma cells intraperitoneally into pristane (Aldrich Chemical Co.)-treated BALB/c mice to stimulate production of ascites fluid. Preinoculation serum specimens were obtained from all mice used for ascites production.

Solid-phase RIA. Polyvinyl chloride microtiter plates were precoated with hyperimmune rotavirus capture antibody (no. 930 or 5192) diluted in PBS, pH 7.4 (18, 34). After an overnight incubation at room temperature, plates were washed three times, and 50 μl of rotavirus antigen was added to the microtiter wells and allowed to bind to the solid phase overnight at room temperature. In some instances, rotavirus antigens were added directly to microtiter plates without prior antibody precoating. Plates coated directly with rotavirus antigens functioned well in solid-phase RIA. In all cases the rotavirus antigens consisted of crude cell culture harvests. After the overnight incubation with antigen, the plates were washed four times with PBS and monoclonal fluid (50 μl) was added to appropriate wells. Plates were incubated for 1 to 2 h at 37°C and again washed four times with PBS. A 50-μl portion of ^{125}I-labeled goat anti-mouse immunoglobulin G, Fc specific or heavy- and light-chain specific (Cappell Laboratories), 100,000 cpm well, was added to the microtiter plate. The antiserum was iodinated by a modification of the Hunter-Greenwood reaction (26). After an additional 1-h incubation at 37°C, plates were washed four times, and individual wells were cut with scissors and counted in a gamma counter. Negative controls consisted of wells to which virus was not added and wells to which NS-1 cell supernatant medium was added. The two types of negative control wells gave similar results. Wells with three times as many counts bound as negative controls were considered positive (positive/negative ≧ 3) (11).

HA and HI assay. Viral hemagglutinins used for the HI assay (see Tables 1 and 2) were prepared by freezing and thawing rotavirus-infected MA104 cells when the cultures exhibited complete or almost complete cytopathic effect. Culture fluid was extracted once with an equal volume of Genetron 113 (Allied Chemical Corp.) and then spun at 2,000 rpm for 15 min. The crude viral extract in the aqueous phase was stored at 4°C and used as HA antigen for HI assay. HI assay was performed as previously described, using 2 to 4 U of hemagglutinin and human O or rhesus monkey erythrocytes (17). Monoclonal antibodies were titrated by serial twofold dilution in PBS with 0.3% bovine serum albumin (17).

Preparation of radiolabeled infected cell lysates. Viral proteins were labeled by using a modification of the method of Lee et al. (22). MA104 monolayers were infected with virus (RRV) at a multiplicity of infection varying from 1 to 10. Virus was initially treated with trypsin (5 μg/ml for 1 h at 37°C). After a 1-h adsorption period, monolayers were washed and refed with Eagle MEM with glutamine and antibiotics. Five to 8 h postinfection the cells were again washed and refed with MEM without methionine. Forty-five minutes later the cells were again washed once and refed with Eagle MEM, without methionine, supplemented with NaCl (150 mM). In some lysate preparations additional salt was not added. Fifteen minutes later 50 μCi of [^{35}S]methionine (800 to 1,200 Ci/mmol; Amersham Corp.) per ml was added and incubation was continued for another hour. Monolayers were then washed three times with MEM, harvested by freezing once (−70°C) and then lysed with a buffer containing 0.8 M KCl–10 mM Tris-hydrochloride (pH 7.8)–1 mM phenylmethylsulfonyl fluoride–1% Triton X-100 (100 μl of buffer per 4×10^5 cells) followed by the same buffer without KCl (400 μl per 4×10^5 cells). The rotavirus-infected cell lysate was then centrifuged for 2 h at 40,000 rpm (SW40 rotor) and stored before use at −70°C. Tunicamycin (5 μg/ml)-treated, [^{35}S]methionine-labeled, RRV-infected cell lysates were prepared in the same fashion except that tunicamycin was present throughout the viral infection.

Immunoprecipitation. A 50- or 100-μl portion of lysate was added to 50 or 100 μl of hybridoma culture fluid supernatant. Then, 300 μl of PBS was added to the reaction, and the mixture was incubated at 37°C for 1 h followed by 4°C overnight. When mouse ascites was used for immunoprecipitation, 5 or 10 μl was added to the reaction mixture. A 50-μl amount of 10% staphylococcus A cells (Pansorbin; Calbiochem) or 50 μl of a 50% slurry of staphylococcus A protein conjugated to Sepharose 4B (Pharmacia Fine Chemicals, Inc.) was then added, and the mixture was incubated for 15 to 30 min. The bacterial suspension or Sepharose was then pelleted (10,000 rpm in an Eppendorf microfuge) and washed three times in 500 μl of RIPA buffer (0.05 M Tris, pH 7.5, 0.2 M NaCl, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 0.1% Nonidet P-40, 0.1% Triton X-100, 0.001 M phenylmethylsulfonyl fluoride). Bound, labeled proteins were removed from the staphylococcus A by boiling for 5 min in 50 μl of solubilizing mixture (0.06 M Tris, pH 6.8, 5% mercaptoethanol, 2% sodium dodecyl sulfate). The bacterial suspension or Sepharose was again pelleted by centrifugation, and the supernatants were applied to 12% sodium dodecyl sulfate-polyacrylamide gels as previously described (11).

Neutralization assay. Supernatant fluids from hybridoma cultures, ascites from mice inoculated with selected hybridomas, and preinoculation mouse sera were titrated in a standard 60% plaque reduction assay as previously described (32). A 200-μl portion of virus activated with trypsin and representing 15 to 80 PFU was mixed with 200 μl of serial two- or fourfold dilutions (in MEM) of monoclonal antibody. After a 1-h incubation period at 37°C, the mixture was inoculated onto MA104 monolayers and allowed to adsorb for 1 h. Wells were then washed once and overlaid with Eagle MEM-agarose as described before (32). After 3 to 6 days, an agarose overlay containing neutral red was added and the plaques were counted. Negative controls included virus mixed with NS-1 supernatant fluid, virus incubated with monoclonal culture fluid (HAT), and virus mixed with preinoculation mouse serum.

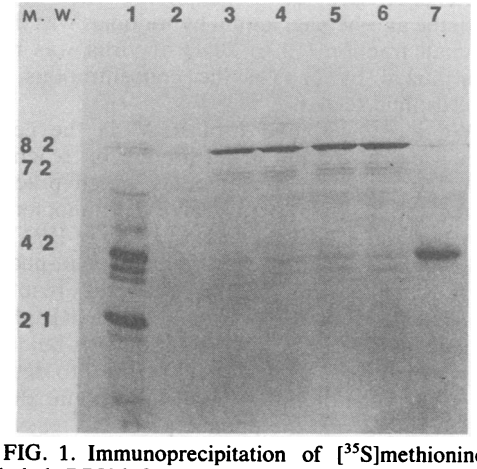

FIG. 1. Immunoprecipitation of [^{35}S]methionine-labeled RRV-infected cell lysate by four representative monoclonal antibodies (lanes 3 to 6) with HI activity against RRV. Lane 7 shows a control immunoprecipitation with a monoclonal antibody directed at the 42-kd major internal structural protein of RRV. Immunoprecipitations were done with cell culture supernatants as described in the text. Lane 1, RRV-infected cell lysate; lane 2, immunoprecipitation with NS-1 cell supernatant; lane 3, immunoprecipitation with monoclonal antibody 954/155/43, sister clone of 954/154/25; lane 4, immunoprecipitation with monoclonal antibody 954/145/55; lane 5, immunoprecipitation with monoclonal antibody 954/23/4; lane 6, immunoprecipitation with monoclonal antibody 954/177/14; lane 7, immunoprecipitation with monoclonal antibody 255/60, which precipitates the gene 6 product (11). Molecular weight (M.W.; × 10^3) is shown on the left.

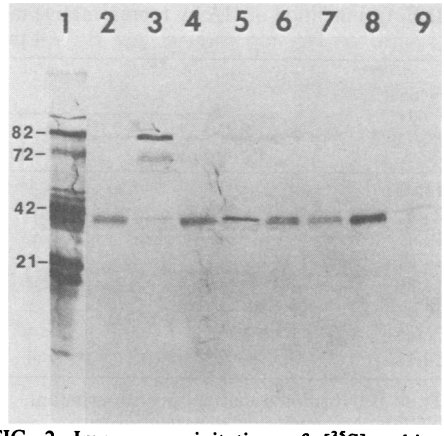

FIG. 2. Immunoprecipitation of [^{35}S]methionine-labeled RRV-infected cell lysate by two monoclonal antibodies (lanes 2, 4, 6, 7, 8) with HI activity and high-titer neutralization activity against RRV. Lane 3 shows Immunoprecipitation of the 82-kd viral hemagglutinin, and lane 5 shows immunoprecipitation of the 42-kd major internal structural protein of RRV. Immunoprecipitations were done with mouse ascites fluid as described in the text. Lane 1, RRV-infected cell lysate; lane 2, immunoprecipitation with monoclonal antibody 954/159/33; lane 3, immunoprecipitation with monoclonal antibody 954/23/4; lane 4, immunoprecipitation with monoclonal antibody 954/96/18; lane 5, immunoprecipitation with monoclonal antibody 631/24, which precipitates the sixth gene product (11); lane 6, immunoprecipitation with monoclonal antibody 954/159/33; lane 7, immunoprecipitation with monoclonal antibody 954/159/13, sister clone of 954/159/33; lane 8, immunoprecipitation with monoclonal antibody 954/96/23, sister clone of 954/96/18; lane 9, immunoprecipitation with control ascites. Molecular weight (×10^3) is shown on the left.

RESULTS

Previous studies in our laboratory had shown that screening of monoclones by solid-phase RIA led to the preferential selection of hybridomas directed at the major inner structural protein of rotaviruses, the 42-kd product of gene 6 (11). We chose, therefore, to use an HI assay as the primary screening procedure for the isolation of monoclonal antibodies directed at the

TABLE 1. Inhibition of HA by representative monoclonal antibodies directed against the 82-kd protein (gene 4 product) of RRV

Monoclonal antibody designation	HI titer (reciprocal)[a] vs given virus (species of origin)				
	RRV (simian)	SA-11 (simian)	CU-1 (canine)	NCDV (bovine)[b]	OSU (porcine)
954/155/25	32	<4	<4	<4	<4
954/23/4	128	16	<4	<4	4
954/145/55	128	32	<4	<4	8
954/177/14	64	8	<4	<4	<4
952/17/36	≧256	<4	<4	<4	<4
952/167/34	32	<4	<4	<4	4
952/266/51	≧256	<4	<4	<4	<4
952/24/18	32	<4	<4	<4	<4

[a] Titer of hybridoma cell culture supernatant.
[b] NCDV, Neonatal calf diarrhea virus.

surface proteins of RRV. From 1,000 wells initially screened by HI, 39 separate monoclonal antibodies with HI activity were eventually cloned and grown up in volume for further analysis. These monoclones formed the basis of the further studies reported here.

Thirty-six of the 39 monoclonal antibodies with HI activity precipitated an 82-kd protein from RRV cell lysates (Fig. 1, lanes 3, 4, 5, and 6, and Fig. 2, lane 3). In addition, these monoclonal antibodies precipitated one to three less intense bands in the 70- to 75-kd range (Fig. 1 and 2). The monoclonal antibodies which precipitated the 82-kd protein were titrated by HI against a variety of hemagglutinating rotaviruses. Representative data from eight of these hybridomas are shown in Table 1. Each exhibited a high degree of homologous specificity in the HI assay. Low-level cross-reactivity with monkey SA-11 and porcine OSU rotaviruses was observed in a few instances (Table 1, monoclone 954/145/55, for example). The monoclonal antibodies which immunoprecipitated the 82-kd protein were also assayed in a standard plaque reduction neutralization assay against RRV. Neutralizing activity was not detected in the supernatants of these monoclonal cell cultures tested at a 1:20 dilution. Because of the lack of detectable neutralizing activity in the hybridoma cell culture supernatant fluids, ascites fluids derived from mice inoculated with monoclonal antibodies to the 82-kd protein were tested by plaque reduction neutralization. The HI titers versus RRV of the ascites fluid were between 10^2- and $10^{3.5}$-fold higher than titers of the culture fluid supernatant. Interestingly, moderate to high levels of neutralizing activity were detected when the ascites fluid was studied in a plaque reduction neutralization assay (Table 2). The preinoculation serum-neutralizing antibody titer of each of the mice against RRV was <1:500. In limited neutralization tests against heterologous viruses, the neutralizing activity appeared to be highly specific for the immunizing RRV strain (Table 2, monoclone 954/23/4, for example). The neutralizing activity of these monoclonal antibodies was not affected by trypsin treatment of RRV at a concentration that enhances infectivity (data not shown). A peculiar characteristic of the plaque reduction assay with the anti-hemagglutinin hybridomas was that a small fraction (10 to 20%) of virus was not neutralized by any of the concentrations of ascites fluid tested.

We identified gene 4 of RRV as the gene which coded for the 82-kd protein by reacting selected monoclonal antibodies which precipitated the 82-kd protein (Table 3, monoclones 954/23/4 and 952/17/36) with a variety of RRV × UK bovine rotavirus reassortants in solid-phase RIA. Since these monoclonal antibodies reacted specifically with RRV in solid-phase RIA but failed to bind to UK bovine virus, their binding to selected reassortants could be used to determine the gene origin of the 82-kd protein. Both the genotype and the phenotype of the reassortants studied had previously been determined (15). The UK bovine rotavirus parent does not hemagglutinate erythrocytes whereas RRV does (15). Reassortants with a gene 4 derived from the RRV parent hemagglutinated erythrocytes, whereas those reassortants in which gene 4 was derived from the UK bovine rotavirus did not hemagglutinate. The monoclonal antibodies which precipitated the 82-kd protein (Table 3, monoclones 954/23/4 and 952/17/36) reacted in RIA only with those reassortants which contained a hemagglutinating protein, that is, reassortants that derived their fourth gene from RRV. All other rotaviral genes could be excluded as coding for the protein which reacted with these monoclonal antibodies.

TABLE 2. Plaque reduction neutralization titers of representative monoclonal antibodies directed against the 82-kd protein (gene 4 product) of RRV

Monoclonal antibody designation	Neutralization titer[a] (reciprocal) vs given virus (species of origin)					
	RRV (simian)	CU-1 (canine)	SA-11 (simian)	OSU (porcine)	UK (bovine)	Wa and DS-1 (human)
954/155/25	≧204,800	<500	<500	<500	<500	<500
954/23/4	≧204,800	<500	2,000	<500	<500	<500
954/145/55	51,200	ND[b]	ND	ND	ND	ND
954/177/14	≧204,800	ND	ND	ND	ND	ND
952/17/36	52,200	ND	ND	ND	ND	ND
952/167/34	204,800	ND	<500	ND	ND	ND
952/266/51	204,800	ND	ND	ND	ND	ND
952/24/8	3,200	ND	ND	ND	ND	ND

[a] Titer of mouse ascites fluid. Preinoculation titer of mouse serum was <500 in all cases.
[b] ND, Not done.

Three monoclonal antibodies had HI activity (Table 4) but did not precipitate the 82-kd protein (Fig. 2, lanes 4, 6, 7, and 8, and Fig. 3, lane 5). These three monoclonal antibodies immunoprecipitated a 38-kd band that migrated just ahead of the 42-kd major inner structural protein of RRV (Fig. 2 and 3). Experiments with tunicamycin-treated cell lysates demonstrated that this band was glycosylated and that the non-glycosylated precursor protein was precipitated by the monoclonal antibodies in the tunicamycin-treated lysate (Fig. 3, lanes 6 and 8). Cell culture supernatant fluid from these three monoclonal antibodies inhibited HA by the RRV, canine rotavirus, and to a lesser extent SA-11 (Table 4). Unlike the hybridomas directed at the 82-kd protein, monoclonal cell culture supernatant fluid from these three hybridomas exhibited neutralizing activity against RRV (titer, 1:160 to 1:320; data not shown). Mouse ascites fluid derived from the three monoclonal antibodies (Table 5) exhibited high-titer neutralizing activity against the homologous RRV. Unlike the monoclonal antibodies to the 82-kd protein, these monoclonal antibodies also efficiently neutralized the serologically related canine and SA-11 rotaviruses. The P strain of human rotavirus, which is also serologically related to RRV (R. Wyatt, unpublished data), was neutralized by two of the three monoclonal antibodies (Table 5). The serologically distinct bovine UK and human Wa strains were not neutralized. At dilutions of 1:2,000 or less the three monoclones to the 38-kd glycoprotein effected complete neutralization of RRV (100% plaque reduction).

Since the monoclonal antibodies which immunoprecipitated the 38-kd glycoprotein reacted specifically with RRV and not UK rotavirus in solid-phase RIA (Table 3, monoclones 954/159/33 and 954/96/18), we could use the same strategy to make gene-coding assignments for these monoclones as we had used for the monoclonal antibodies which immunoprecipitated the 82-kd protein. In previous studies we had shown that viral serotype was primarily a function of the eighth or ninth RNA segment, whereas viral hemagglutinin was coded for by the fourth RNA segment (9, 15, 16). When tested in solid-phase RIA, the monoclonal antibodies against the 38-kd glycoprotein bound only to RRV × UK bovine rotavirus reassortants that were serotypically RRV, whether or not the reassortant hemagglutinated erythrocytes (Table 3, monoclones 954/159/33 and 954/96/18). When the genotype of the reassortants was correlated with the monoclonal binding in solid-phase RIA, it was found that monoclonal antibody binding correlated with the presence in the reassortant of an eighth or ninth gene derived from RRV. These two genes could not be differentiated in the reassortants. These data indicated that the 38-kd glycoprotein is the product of one of these two gene segments.

Because the monoclonal antibodies directed at the 38-kd glycoprotein had HI activity but did not appear to bind to the viral hemagglutinin in ^{35}S-labeled cell lysates or in solid-phase RIA, we tested these monoclonal antibodies for their capacity to inhibit HA by selected reassortants (Table 6). Monoclonal antibodies 954/145/55 and 952/147/3, which reacted with the 82-kd protein and tracked to the fourth gene product in solid-phase RIA, had HI activity against all reassortant viruses irrespective of the reassortant neutralization serotype (bovine or simian). On the other hand, monoclonal antibodies 954/159/33 and 952/3/68, which precipitated the 38-kd protein and tracked to the eighth or ninth RRV gene product in RIA, had HI activity only when the reassortants exhibited the rhesus rotavirus neutralization serotype. The RRV HA activity of reassortants 28-1 and 85-2, which had the bovine

TABLE 3. Solid-phase RIA binding assay of selected monoclonal antibodies to antigens of reassortants derived from bovine (UK) × RRV mixed infection

Monoclonal antibody designation[a]	Positive/negative ratio of monoclonal antibodies bound to given rotavirus reassortant[b]						
	28-1 (UK/HA) 3, 4, 8 or 9, 11	9-1 (RRV/no HA) 2, 7, 8 or 9, 11	86-1 (RRV/HA) 2, 4, 7, 8, 9, 10, 11	85-2 (UK/HA) 2, 3, 4, 7, 8 or 9, 10, 11	8-1-2 (UK/no HA) 2, 11	RRV[c] (RRV/HA) 1 through 11	UK[c] (UK/no HA)
954/23/4	*3.5*[d]	0.9	*5.4*	*6.4*	1.0	*6.8*	0.9
952/17/36	*17*	0.7	*12*	*12.0*	0.8	*12*	0.6
954/159/33	1.6	*51*	*28*	1.7	2.3	*46*	2.3
954/96/18	2.3	*19*	*11*	2.2	1.4	*15*	1

[a] Monoclones 954/23/4 and 952/17/36 immunoprecipitate an 82-kd RRV protein. Monoclones 954/159/33 and 954/96/18 immunoprecipitate and 38-kd RRV glycoprotein.

[b] Phenotypes of each reassortant (neutralization/HA) and genes derived from the RRV parent are indicated.

[c] RRV hemagglutinates human O erythrocytes; UK bovine rotavirus does not hemagglutinate erythrocytes. Reassortant rotaviruses (UK × RRV) with fourth genes derived from RRV hemagglutinate. Monoclonal antibodies 954/23/4 and 952/17/36 react only with the reassortants in which gene 4 is derived from RRV. Monoclonal antibodies 954/159/33 and 954/96/18 react only with reassortants in which gene 8 or 9 is derived from RRV.

[d] Italics indicate significant binding; positive/negative ratio ≧ 3.5 as described in the text.

serotype, was not inhibited by these monoclonal antibodies (Table 6, monoclones 954/159/33 and 952/3/68). This suggests that the HI activity of these monoclonal antibodies was not mediated by direct interaction with the hemagglutinin.

DISCUSSION

We have recently shown by a genetic analysis of rotavirus reassortants that gene 4 of RRV codes for the viral hemagglutinin. In addition, the fourth gene product was associated with protease-enhanced plaque formation, whereas gene 8 or 9 coded for the primary neutralization determinant (15). In the present work we have used monoclonal antibodies directed at two surface proteins of RRV to confirm and extend these findings. Because of previous difficulty in isolating monoclonal antibody to the outer capsid proteins of rotavirus, in these experiments we chose to use an HI test rather than RIA as our principal screening technique. With this strategy we were able to isolate and clone 39 separate hybridomas with a high level of HI activity. The majority of these monoclonal antibodies immunoprecipitated an 82-kd protein that could be shown to be the product of gene 4 of RRV. In addition, most of these monoclonal antibodies immunoprecipitated several smaller proteins (Fig. 1 and 2) which are presumably tryptic cleavage products of the 82-kd primary gene product (5, 6). The lysate used in these studies was harvested in the presence of a trypsin inhibitor, phenylmethylsulfonyl fluoride. Inhibition, however, appears to have been incomplete. The putative cleavage products seen in the RRV lysate are somewhat larger than those described by other workers (5, 6). Of interest, none of the monoclonal antibodies precipitated a protein in the 20- to 30-kd range. Other workers have shown that the primary product of gene 4 is cleaved in vitro and in vivo into two pieces, a larger fragment and a smaller fragment in the 20- to 30-kd range (5, 6). Since our monoclonal antibodies were selected for their ability to inhibit HA, it is possible that this smaller cleavage fragment does not contain the HA domain of the molecule and hence was not immunoprecipitated.

The finding that ascites fluid containing high levels of monoclonal antibody directed at the 82-kd protein, the fourth gene product, had substantial neutralizing activity is of interest. Despite the demonstration by Hayes et al. (13) that several reovirus proteins can mediate viral neutralization, a similar finding with rotavirus was unexpected. Previous genetic studies of reassortants derived from human rotavirus strains Wa, W, and DS-1 had not demonstrated neutralizing activity segregating in association with gene 4 (9, 10, 16). However, in these studies gene 4 of the human rotavirus could not be evaluated since it was not present in any of the cultivable reassortants (9, 10, 12, 16). In addition, the antiserum used to characterize the UK virus and to select serotypic human reassortants came from guinea pigs immunized with the bovine Nebraska calf diarrhea virus. In retrospect it seems likely and fortunate that this antiserum, although highly cross-reactive with the UK bovine 38-kd glycoprotein, contained little or no neutralizing activity directed at the UK gene 4 product. Presumably the gene 4 products of these two bovine viruses are not related antigenically. A recent analysis of UK bovine rotavirus × RRV reassortants demonstrated that infectivity was probably neutralized by antibodies directed against the

gene 4 product. In these studies, however, the primary neutralizing activity of the hyperimmune sera was clearly directed at the gene 8 or 9 product (15). Killen and Dimmock have prepared monospecific hyperimmune antisera to several UK bovine rotavirus proteins which were isolated individually from polyacrylamide gels (21). Antisera to VP 4.2, presumably the gene 4 product, did not neutralize UK rotavirus. However, in these studies denatured protein was used as an immunogen and the antiserum produced was not potent since it failed to immunoprecipitate labeled viral protein from a cell lysate, gave only ± immunofluorescence, and did not aggregate virus by immune electron microscopy.

Burstin et al. demonstrated the σ_1 protein of reovirus contains several functional domains including epitopes involved in HA only, in neutralization only, in both functions, or in neither (3). Spriggs and Fields presented indirect evidence that only the neutralization domain of σ_1 was involved in neurotropism of reovirus (28). The monoclonal antibodies directed at the rotavirus 82-kd protein all appear to be directed at antigenic domains with shared neutralization and HA activity. Whether these areas of the 82-kd protein are involved in tissue or host tropism remains to be determined. These monoclonal antibodies are apparently directed at a site distinct from the trypsin cleavage site, since neither inhibition of HA nor neutralization of the virus by gene 4 monoclonal antibodies appears to be affected by proteolytic activation of virus. Interestingly, monoclonal antibodies directed at the reovirus protein that has a biologically active proteolytic cleavage site (μlc) do not neutralize infectivity (13).

The three monoclonal antibodies directed to the 38-kd RRV glycoprotein each neutralized the virus to high titer. This was to be expected since previous studies by Bastardo et al., Killen and Dimmock, and Matsuno and Inouye had shown that hyperimmune monospecific antiserum to the 38-kd glycoprotein neutralizes rotavirus (1, 21, 24). The demonstration that this protein is the product of the eighth or ninth gene confirms our genetic data (15) and supports the translation studies of others (23, 25, 27). Despite the fact that the 38-kd glycoprotein is not the viral hemagglutinin, monoclonal antibody directed at it exhibited HI activity. Similar findings were made by Bastardo et al., using polyvalent antisera to SA-11, and by Matsuno and Inouye, using polyvalent antisera to neonatal calf diarrhea virus (1, 24). Both of these authors interpreted these findings as indicating that the 38-kd glycoprotein was the rotavirus hemagglutinin. We could demonstrate, however, that the HI activity of the hybridomas directed at the 38-kd glycoprotein was not a function of direct interaction with the 82-kd hemagglutinin since these monoclonal antibodies failed to inhibit HA of reassortants with a bovine serotype and an RRV hemagglutinin (Table 6, monoclones 954/159/33 and 952/3/68). Probably, as is the case with antibodies to the influenza neuraminidase, HA

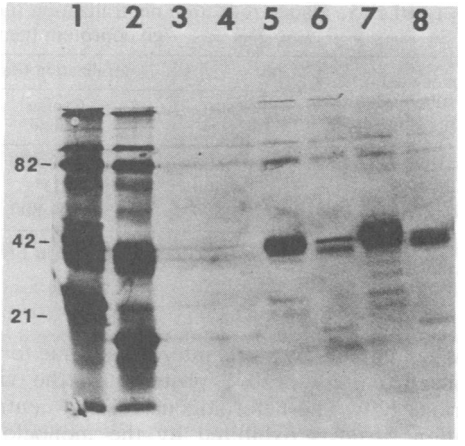

FIG. 3. Immunoprecipitation of [^{35}S]methionine-labeled RRV-infected cell lysate grown in the presence (+) or absence (−) of tunicamycin as described in the text. Monoclonal antibody 954/96/31 (lanes 5 and 6) precipitates the 38-kd RRV protein and has HI and neutralizing activity. Monoclonal antibody 60/46 (lanes 7 and 8) precipitates the 38-kd protein of many mammalian rotaviruses but does not have HI or neutralizing activity. Lane 1, RRV-infected lysate (−); lane 2, RRV-infected lysate (+); lane 3, RRV-infected lysate (−) immunoprecipitated with HAT fluid; lane 4, RRV-infected cell lysate (+) immunoprecipitated with HAT fluid; lane 5, RRV-infected cell lysate (−) immunoprecipitated by monoclonal antibody 954/96/31, sister clone of 954/96/18 and /23; lane 6, RRV-infected cell lysate (+) immunoprecipitated by 954/96/31; lane 7, RRV-infected cell lysate (−) immunoprecipitated by monoclonal antibody 60/46 raised to the Wa rotavirus 38-kd glycoprotein (unpublished data); lane 8, RRV-infected cell lysate (+) immunoprecipitated by monoclonal antibody 60/44. Molecular weight ($\times 10^3$) is shown on the left.

TABLE 4. Inhibition of HA by representative monoclonal antibodies directed against the 38-kd glycoprotein (gene 8 or 9 product) of RRV

Monoclonal antibody designation	HI titer (reciprocal)[a] with given virus (species of origin)				
	RRV (simian)	SA-11 (simian)	CU-1 (canine)	NCDV (bovine)[b]	OSU (porcine)
954/159/33	80	160	320	<4	<4
952/3/68	40	<4	160	<4	<4
954/96/18	40	<4	40	<4	<4

[a] Titer of hybridoma cell culture supernatant fluid.
[b] NCDV, Neonatal calf diarrhea virus.

TABLE 5. Plaque reduction neutralization titers of monoclonal antibodies directed against the 38-kd glycoprotein (gene 8 or 9 product) of RRV

Monoclonal antibody designation	Neutralization titer (reciprocal)[a] vs given virus (species of virus origin)				
	Rhesus (simian)	Canine (canine)	SA-11 (simian)	P (human)	UK, Wa (bovine, human)
954/159/33	≧204,800	102,400	≧204,800	≧16,000	<200
954/96/18	≧204,800	ND[b]	ND	<500	<200
952/3/68	≧204,800	≧204,800	12,800	≧16,000	<200

[a] Titer of mouse ascites fluid. Preinoculation titer of mouse serum was <1/200 in all cases.
[b] ND, Not done.

can be inhibited by steric interference due to the close proximity of two proteins on the viral surface (20). The heterologous HI and neutralization patterns exhibited by the monoclonal antibodies to the 38-kd glycoprotein were similar to observations made with hyperimmune sera and clearly differed from data derived with monoclonal antibodies to the gene 4 product (canine rotavirus, Tables 1, 2, 4, and 5, for example). In the future, analysis of rotavirus HI and neutralization assays will have to take note of the fact that antibodies to two separate proteins are being measured in a single assay.

The number of functional epitopes of the 38-kd glycoprotein was not investigated by us and will require the production of more monoclonal antibodies for thorough analysis. In unpublished studies we have isolated several monoclonal antibodies from mice immunized with Wa human rotavirus which cross-react with virtually all mammalian rotaviruses in solid-phase RIA.

TABLE 6. HI antibody titer of selected monoclones[a]

Monoclonal antibody[b]	HI titer (reciprocal) of monoclonal antibody vs given reassortant (serotype of reassortant)			
	28-1 (UK)[c]	86-1 (RRV)	48-2 (RRV)	85-2 (UK)
954/145/55	≧16	≧16	≧16	≧16
952/147/3	≧16	≧16	≧16	NT
954/159/33	<4	≧16	≧16	<4
952/3/68	<8	≧16	≧16	NT

[a] Bovine (UK) × rhesus (RRV) rotavirus reassortants were used as HA antigens in the assay.
[b] Monoclonal antibodies 954/145/55 and 952/147/3 immunoprecipitate the 82-kd protein, the gene 4 product, the viral hemagglutinin. Monoclonal antibodies 954/159/33 and 952/3/68 immunoprecipitate the 38-kd glycoprotein, the gene 8 or 9 product, the protein which determines virus serotype.
[c] Genotype of the indicated reassortant. Genes derived from the RRV parent are as follows: 28-1—3, 4, 8 or 9, 11; 86-1—2, 4, 7, 8 or 9, 10, 11; 48-2—2, 4, 8 or 9, 10, 11; 85-2—2, 3, 4, 7, 8 or 9, 10, 11. Gene 4 of all reassortants is derived from RRV.

These monoclonal antibodies immunoprecipitate the 38-kd glycoprotein of Wa virus, as well as those of RRV and other mammalian rotaviruses (Fig. 3, lanes 7 and 8). These same monoclonal antibodies do not neutralize Wa or RRV or inhibit RRV HA, and presumably they define a separate epitope on the rotavirus glycoprotein not involved in these functions. This epitope, in contrast to the epitope involved in virus neutralization, is antigenically cross-reactive among most mammalian rotaviruses.

The biological functions of several of the rotavirus outer capsid proteins in vitro are beginning to come into focus; however, the role of these proteins in vivo remains unexplored. It is interesting that both surface proteins exhibit type specificity. Perhaps the production of additional hybridomas will allow definition of epitopes with broader cross-reactivity on the two proteins. The relative importance to the host of an immune response to both the 82- and 38-kd proteins requires careful analysis in animal models and in humans. The mechanism of viral neutralization as mediated through the two proteins might well be different. Certainly it is not clear which of these two surface proteins (or both) is the primary determinant of tissue tropism and host range. Additional studies need to be done to determine which protein is primarily involved in cell binding and which is involved in cell penetration or exit from lysosomal vacuoles. The availability of specific monoclonal antibodies should aid in elucidating some of these questions.

ACKNOWLEDGMENTS

We thank Louis Potash, Lee Cline, and Ronald Jones for technical assistance. We thank Robert Chanock and Albert Kapikian for advice and encouragement. Finally, we thank Linda Jordan for editorial assistance.

LITERATURE CITED

1. **Bastardo, J. W., J. L. McKimm-Breschkin, S. Sonza, L. D. Mercer, and I. H. Holmes.** 1981. Preparation and characterization of antisera to electrophoretically purified SA-11 virus polypeptides. Infect. Immun. **34**:641–647.
2. **Bridger, J. D., and G. N. Woode.** 1975. Neonatal calf diarrhoea: identification of a reovirus-like (rotavirus) agent in faeces by immunofluorescence and immune elec-

tron microscopy. Br. Vet. J. 131:528–535.
3. **Burstin, S. J., D. R. Spriggs, and B. N. Fields.** 1982. Evidence for functional domains on the reovirus type 3 hemagglutinin. Virology 117:146–155.
4. **Cohen, J.** 1977. Ribonucleic acid polymerase activity associated with purified calf rotavirus. J. Gen. Virol. 36:395–402.
5. **Espejo, R. T., S. Lopez, and A. Carlos.** 1981. Structural polypeptides of simian rotavirus SA-11 and the effect of trypsin. J. Virol. 37:156–60.
6. **Estes, M. K., D. Y. Graham, and B. B. Mason.** 1981. Proteolytic enhancement of rotavirus infectivity: molecular mechanisms. J. Virol. 39:879–888.
7. **Flewett, T. H., and G. N. Woode.** 1978. The rotaviruses, a brief review. Arch. Virol. 57:1–23.
8. **Flores, J., H. B. Greenberg, J. Myslinski, A. R. Kalica, R. G. Wyatt, A. Z. Kapikian, and R. M. Chanock.** 1982. Use of transcription probes for genotyping rotavirus reassortants. Virology 121:288–295.
9. **Greenberg, H., J. Flores, A. Kalica, R. Wyatt, and R. Jones.** 1983. Gene coding assignments for growth restriction, neutralization and subgroup specificities of the W and DS-1 strains of human rotavirus. J. Gen. Virol. 64:313–324.
10. **Greenberg, H. B., A. R. Kalica, R. G. Wyatt, R. W. Jones, A. Z. Kapikian, and R. M. Chanock.** 1981. Rescue of noncultivatable human rotavirus by gene reassortment during mixed infection with *ts* mutants of a cultivatable bovine rotavirus. Proc. Natl. Acad. Sci. U.S.A. 78:420–424.
11. **Greenberg, H. B., V. McAuliffe, J. Valdesuso, R. Wyatt, J. Flores, A. Kalica, Y. Hoshino, and N. Singh.** 1983. Serological analysis of the subgroup protein of rotavirus, using monoclonal antibodies. Infect. Immun. 39:91–99.
12. **Greenberg, H. B., R. G. Wyatt, A. Z. Kapikian, A. R. Kalica, J. Flores, and R. Jones.** 1982. Rescue and serotypic characterization of noncultivatable human rotavirus by gene reassortment. Infect. Immun. 37:104–109.
13. **Hayes, E. C., P. W. K. Lee, S. E. Miller, and W. K. Joklik.** 1981. The interaction of a series of hybridoma IgGs with reovirus particles. Virology 108:147–155.
14. **Hoshino, Y., R. G. Wyatt, F. W. Scott, and M. J. Appel.** 1982. Isolation and characterization of a canine rotavirus. Arch. Virol. 72:113–125.
15. **Kalica, A. R., 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.
16. **Kalica, A. R., H. B. Greenberg, R. G. Wyatt, J. Flores, M. Sereno, A. Z. Kapikian, and R. M. Chanock.** 1981. Genes of human (strain Wa) and bovine (strain UK) rotaviruses that code for neutralization and subgroup antigens. Virology 112:385–390.
17. **Kalica, A. R., H. D. James, and A. Z. Kapikian.** 1978. Hemagglutination by simian rotavirus. J. Clin. Microbiol. 7:314–315.
18. **Kapikian, A. Z., R. H. Yolken, H. B. Greenberg, R. G. Wyatt, A. R. Kalica, R. M. Chanock, and H. W. Kim.** 1979. Gastroenteritis viruses, p. 927–995. *In* E. Lennette and N. Schmidt (ed.), Diagnostic procedures for viral rickettsial and chlamydial infections. American Public Health Association, Washington, D.C.
19. **Kennett, R. H., T. J. McKearn, and K. B. Bechtol.** 1981. Monoclonal antibodies, p. 365–366. *In* Hybridomas: a new dimension in biological analyses. Plenum Publishing Corp., New York.
20. **Kilbourne, E.** 1968. Recombination of influenza A viruses of human and animal origin. Science 160:74–75.
21. **Killen, H. M., and N. J. Dimmock.** 1982. Identification of a neutralization-specific antigen of a calf rotavirus. J. Gen. Virol. 62:297–311.
22. **Lee, P. W., E. C. Hayes, and W. K. Joklik.** 1981. Characterization of anti-reovirus immunoglobulins secreted by cloned hybridoma cell lines. Virology 108:131–146.
23. **Mason, B. B., D. Y. Graham, and M. K. Estes.** 1980. In vitro transcription and translation of simian rotavirus SA-11 gene products. J. Virol. 33:1111–1121.
24. **Matsuno, S., and S. Inouye.** 1983. Purification of an outer capsid glycoprotein of neonatal calf diarrhea virus and preparation of its antisera. Infect. Immun. 39:155–158.
25. **McRae, M. A., and J. G. McCorquodale.** 1982. The molecular biology of rotavirus. II. Identification of the protein-coding assignments of calf rotavirus genome RNA species. Virology 117:435–443.
26. **Purcell, R. H., D. C. Wong, H. J. Alter, and P. V. Holland.** 1973. Microtiter solid-phase radioimmunoassay for hepatitis B antigen. Appl. Microbiol. 26:478–484.
27. **Smith, M. L., E. Lazdins, and I. H. Holmes.** 1980. Coding assignments of double-stranded RNA segments of SA-11 rotavirus established by in vitro translation. J. Virol. 33:976–982.
28. **Spriggs, D. R., and B. N. Fields.** 1982. Attenuated reovirus type 3 strains generated by selection of haemagglutinin antigenic variants. Nature (London) 297:68–70.
29. **Stuker, G., L. S. Oshino, and N. J. Schmidt.** 1980. Antigenic comparisons of two new rotaviruses from rhesus monkeys. J. Clin. Microbiol. 11:202–203.
30. **Theil, K. W., E. H. Bohl, R. F. Cross, E. Kohler, and A. Agnes.** 1978. Pathogenesis of porcine rotaviral infection in experimentally inoculated gnotobiotic pigs. Am. J. Vet. Res. 39:213–220.
31. **Wyatt, R. G., H. B. Greenberg, W. D. James, A. L. Pittman, A. R. Kalica, J. Flores, R. M. Chanock, and A. Z. Kapikian.** 1982. Definition of human rotavirus serotypes by plaque reduction assay. Infect. Immun. 37:110–115.
32. **Wyatt, R. G., and W. D. James.** 1981. Methods of viral culture *in vivo* and *in vitro*, p. 13–36. *In* D. A. J. Tyrrell and A. Z. Kapikian (eds.), Virus Infections of the Gastrointestinal Tract. Dekker, New York.
33. **Wyatt, R. G., A. R. Kalica, C. A. Mebus, H. W. Kim, W. T. London, R. M. Chanock, and A. Z. Kapikian.** 1978. Reovirus-like agents (rotaviruses) associated with diarrheal illness in animals and man. Perspect. Virol. 10:121–145.
34. **Yolken, R. H., B. Barbour, R. G. Wyatt, A. R. Kalica, A. Z. Kapikian, and R. M. Chanock.** 1978. Enzyme-lined immunosorbent assay for identification of rotaviruses from different animal species. Science 201:259–262.