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Neutralizing Monoclonal Antibodies to Human Rotavirus and Indications of Antigenic Drift Among Strains from Neonates

BARBARA S. COULSON,[1]* KERRY J. FOWLER,[2] RUTH F. BISHOP,[1] AND RICHARD G. H. COTTON[2]

Department of Gastroenterology[1] and Birth Defects Research Institute,[2] Royal Children's Hospital, Parkville, Victoria, 3052, Australia

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Cells producing neutralizing monoclonal antibodies to a serotype 3 human neonatal rotavirus strain RV-3 were derived by fusion of hyperimmunized mouse spleen cells with mouse myeloma cells. As ascites fluid, three rotavirus-neutralizing monoclonal antibodies were characterized by hemagglutination inhibition and reacted with 17 cultivable mammalian rotaviruses representing five virus serotypes, by fluorescent focus neutralization and enzyme immunoassay. Two antibodies, Mab RV-3:1 and Mab RV-3:2, reacted with the seven serotype 3 rotaviruses only. Mab RV-3:1 was shown to bind to the outer capsid glycoprotein gp34 of rotavirus when variants of SA 11 rotavirus were used, and it therefore appears to react with the major neutralization epitope of serotype 3 rotaviruses. The antibody Mab RV-3:3 was specific for an epitope of RV-3 rotavirus not present on any other rotavirus of any serotype tested, including another neonatal isolate of identical RNA electropherotype isolated from the same ward of the same hospital as RV-3 3 months earlier. These two viruses were also distinguishable by fluorescent focus neutralization, using antiserum to RV-3 virus. Western blot analysis showed binding of Mab RV-3:3 to the trypsin cleavage product of the outer capsid protein p86 of RV-3. This suggests that antigenic drift may have occurred among neonatal rotaviruses in Melbourne. These monoclonal antibodies will be useful in serotyping assays of rotaviruses directly in stool samples, and in further analysis of antigenic variation within the serotype.

A number of authors have reported production of monoclonal antibodies to mammalian rotaviruses (11, 13, 24). Most of these antibodies were directed to the major inner capsid protein, which bears shared antigenic determinants as well as those involved in subgroup reactions (13). Recently, strain-specific neutralizing monoclonal antibodies directed at surface proteins of the serotype 3 simian rotaviruses SA 11 (27) and RRV-2 (14) have been described. Two distinct epitopes on the major surface glycoprotein of SA 11 rotavirus which relate to virus neutralization were identified with the neutralizing monoclonal antibodies to SA 11 (28). However, the production of neutralizing monoclonal antibodies to human rotaviruses has not been reported.

An important application of rotavirus-neutralizing monoclonal antibodies not yet addressed is the serotyping of rotavirus strains directly from stools, by enzyme immunoassay (EIA) or equivalent method. Rotavirus serotyping has been achieved with EIA (29) and solid-phase immune electron microscopy (12), with carefully cross-absorbed antisera. However, as these antisera are difficult to produce, most serotyping has been by plaque neutralization assays (17, 19, 28, 31) and fluorescent focus neutralization (FFN) assays (3). These methods usually are suitable only for cultivable rotaviruses, so serotyping has been restricted and progress impeded in cross-protection studies and evaluation trials of candidate human rotavirus vaccines.

In this paper, we describe the production and characterization of neutralizing monoclonal antibodies to the neonatal human serotype 3 rotavirus, RV-3 (1). These antibodies were reacted with a panel of mammalian rotaviruses by EIA and FFN assay. The possible occurrence of antigenic drift suggested by the monoclonal antibody reactions with the virus panel was investigated with polyclonal antiserum, and the virus proteins to which the monoclonal antibodies are directed were identified by Western blot analysis and SA 11 rotavirus variants.

* Corresponding author. Previously known as Barbara S. McLean.

MATERIALS AND METHODS

Viruses and cells. Human neonatal rotaviruses RV-1 and RV-3 (serotype 3) and human rotaviruses RV-4 (serotype 1) and RV-5 (serotype 2) were adapted for culture in our laboratory by J. Albert (1). RV-1 and RV-3 were used at passage levels 11 and 13, respectively. Human rotavirus strains YO, S2, and KU were donated by T. Kutsuzawa (31). MMU18006, rhesus rotavirus strain 2 (RRV-2) was the gift of N. Schmidt, and simian rotavirus SA11 was kindly supplied by H. Malherbe (San Antonio, Tex.). Canine rotavirus LSU-79C36 (K9; 10) was provided by G. Woode, who, with J. Bridger, isolated the UK strain of bovine rotavirus provided by T. Flewett (33). Human rotavirus strains Wa, DS-1, P, and ST-3 were kindly donated by R. G. Wyatt.

All viruses were propagated in MA104 cells in the presence of trypsin, as previously described (25).

The myeloma cell line P3-X63-Ag8-653 (clone 653) used for fusion is an azaguanine-resistant derivative of a nonsecreting line of myeloma cell clones (18). The cells were cultured as described previously (6) in Dulbecco modified Eagle medium (DMM) with 20% (vol/vol) heat-inactivated fetal calf serum.

Immunization. BALB/c mice 8 weeks old were immunized with intact, double-shelled RV-3 rotavirus particles, which were prepared by fluorocarbon extraction and banding in cesium chloride gradients as previously described (23). Throughout the virus purification procedure, Tris-buffered saline (pH 7.2) with 10 mM calcium chloride was used to stabilize the outer capsid layer (5). Mice were immunized intraperitoneally with 0.4 ml of virus in Fruend complete adjuvant, and two mice were boosted 2 months later by the same route with 0.1 ml of virus in Freund incomplete

adjuvant, as well as with 0.2 ml of virus by the intravenous route. Three days later, one mouse (A) was sacrificed and its spleen was removed for fusion. The second mouse (B) received a further intravenous boost 2 months after its first boost and was sacrificed for splenic fusion 3 days later. Both mice showed preinoculation anti-RV-3 EIA titers of 1:50 in serum. After the first booster injection, their EIA titers rose to 1:160,000, with RRV-2 hemagglutination inhibition (HI) titers of greater than 1:2,000 and RV-3 FFN titers of 1:50,000.

Production of hybridoma cell lines. The methodology for production of hybridoma cell lines has been described elsewhere (6). Spleen cells from hyperimmunized mice were fused with clone 653 myeloma cells, using polyethylene glycol. Parental myeloma cells were killed by medium containing 100 μM hypoxanthine, 4 nM aminopterin, and 16 μM thymidine. Fused cells were distributed into 96-well (0.2-ml) Linbro plates. The culture fluids were tested for antibody production by EIA, HI, and FFN. Hybridomas that showed HI and FFN activity were subcloned at least twice by limiting dilution and grown in syngeneic mice primed with Pristane (ICN Pharmaceuticals, Plainview, N.J. as ascites.

EIA for rotavirus antibodies. THE EIA was performed as described previously (21). Hybrid culture fluids were tested at a dilution of 1 in 5 in phosphate-buffered saline (PBS) (pH 7.2) containing 1% (wt/vol) bovine serum albumin (PBS-B) in polyvinyl microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) coated with optimal dilutions of RV-3 (serotype 3), RV-5 (serotype 2), and SA 11 virus antigen and MA104 cell control antigen. These EIA antigens were produced by fluorocarbon extraction and ultracentrifugation of virus-infected or uninfected cell harvests as described previously (5, 23). Bound antibody was detected with goat antimouse immunoglobulins conjugated to horseradish peroxidase (DAKO Immunoglobulins, Copenhagen, Denmark) and orthophenylene diamine (Sigma Chemical Co., St. Louis, Mo.) as substrate (32). The absorbance of each well at 492 nm was recorded with a Titertek Multiskan spectrophotometer (Flow Laboratories, Inc., Rockville, Md.). Optimal dilutions of reagents were determined by checkerboard titration.

Ascites fluids and affinity-purified monoclonal antibodies were titrated by EIA, using doubling dilutions of monoclonal antibody. Wells were coated with any of the rotaviruses as described above, and MA104 control antigen, at optimal dilution.

HI assay for rotavirus antibodies. Viral hemagglutinins used for the HI assay were prepared by freezing and thawing rotavirus-infected MA104 cells showing complete cytopathic effect. This virus preparation was stored in aliquots at −70°C. All hybridoma cultures were screened for inhibition of RRV-2 hemagglutination (HA), using PBS-B throughout as diluent. An aliquot (25 μl) of hybrid culture fluid diluted 1 in 5 was incubated at 37°C with an equal volume of HA antigen-containing 4 HA units in U-bottomed microtiter trays. After 1 h, 50 μl of 0.5% human group O erythrocytes was added, and results were recorded after further incubation for 1 h at 37°C. Antigen, cell, and positive and negative hybridoma supernatants and sera were included in each test.

Rotavirus HI antibodies in ascites fluids were titrated in duplicate, using serial twofold dilutions. The HI titers were expressed as the reciprocal of the highest dilution of monoclonal antibody showing complete inhibition of HA.

FFN assay. The FFN assay procedure was based on that of Beards et al. (3) with several modifications, and was used to screen cell hybridoma cultures positive for rotavirus antibody production by EIA. Hybridoma supernatants (25 μl) were diluted 1:1 with DMM containing 1 μg of Sigma porcine trypsin per ml (DMM-T) and then were mixed with an equal volume of RV-3 virus diluted in DMM-T to give ca. 100 fluorescent cell-forming units in 50 μl. The mixtures were incubated at 37°C for 1 h, and 50 μl per well was inoculated in duplicate onto confluent monolayers of MA104 cells in microtiter plates. After centrifugation at 1,200 × *g* for 30 min, the plates were incubated at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity overnight and stained by indirect immunofluorescence.

The dilution of rotavirus RV-3 (serotype 3) giving 100 fluorescent cell-forming units was determined previously by inoculation of MA104 cell monolayers with doubling dilutions of virus in DMM-T, followed by centrifugation, incubation, and staining as described above.

As virus controls, DMM-T and clone 653 supernatant fluid were included. Incubation of clone 653 supernatant alone with RV-3 was found to give a 10% reduction in fluorescent cell count. However, neutralizing hybridoma supernatants reduced the number of fluorescing cells at least 10-fold.

Mouse ascites fluids were titered for neutralizing antibody by using fourfold dilutions of ascites mixed with an equal volume of any of the rotaviruses described above, diluted to give ca. 100 fluorescent cell-forming units per well. The neutralization titer of each fluid was expressed as the reciprocal of the highest dilution giving 50% reduction in the number of fluorescing cells.

Indirect immunofluorescence. Rotavirus-infected cells were fixed in acetone for 5 min and air dried. Rabbit hyperimmune antiserum to SA 11 (50 μl) at optimal dilution (1:500) in PBS was added to each well, and the plates were incubated for 1 h at 37°C. The plates were washed three times with PBS before the addition to each well of 25 μl of fluorescein isothiocyanate-labeled goat antirabbit immunoglobulin G (IgG) (TAGO Inc.) diluted 1:50 in PBS. After a further 1-h incubation at 37°C, the plates were washed again and air dried. The inverted wells were observed with a Zeiss epifluorescent UV microscope (magnification, ×80). Specific fluorescence appeared only in the cytoplasm of infected cells; uninfected cells showed no specific fluorescence.

Plaque neutralization assay. The plaque neutralization assay method of Matsuno et al. (19), modified by Smith et al. (26), was adopted for use with RRV-2 virus by the addition of 50 μg of DEAE-dextran per ml (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) to the first agarose overlay. Hybridoma culture supernatants were screened at a dilution of 1 in 4.

Polyacrylamide gel electrophoresis of double-stranded RNA of rotavirus. Electropherotyping of viral RNA was performed on all stocks of rotavirus strains studied (see Tables 4 and 5). Viral RNA was prepared by phenol-chloroform extraction (8). Polyacrylamide gel electrophoresis of viral RNA was performed with the Laemmli buffer system (26) at 15 mA for 18 h at 4°C. The RNA was visualized with silver stain (8).

Western blot analysis. Partially purified rotavirus particles, prepared by fluorocarbon extraction and ultracentrifugation of infected cell harvests (5, 23), were solubilized in sample buffer and electrophoresed in 9% polyacrylamide-Laemmli gels (26) at 25 mA until the dye front reached 1 cm from the end of the gel. To obtain fully reduced proteins, samples were boiled in sample buffer containing 5% 2-mercaptoethanol and 2% sodium dodecyl sulfate (SDS) and were run in a gel containing SDS. For unreduced proteins, samples were heated to 60°C for 5 min in sample buffer without 2-mercaptoethanol and applied to a gel prepared without SDS. All

TABLE 1. Screening patterns from four fusions of hybridoma clones producing antibody to RV-3

No. of clones detected		No. of clones retained	EIA result with antigen:			HI result	FFN result
Fusions 98 and 99	Fusions 100 and 101		RV-3 (serotype 3)	SA 11 (serotype 3)	RV-5 (serotype 2)		
51	124	10	+	+	+	−	−
1	2	1	+	+	−	−	−
2	1	2	+	+	−	+	+
0	4	1	+	−	−	−	+

running buffers contained SDS. Electrophoresed proteins were transferred electrophoretically to Millipore HAHY nitrocellulose (30) overnight at 30 V, using as transfer buffer 25 mM Tris–192 mM glycine–20% methanol (pH 8.3). Nitrocellulose sheets were probed with antibody by a modification of the method of Burnette (4). The nitrocellulose sheets were soaked in 10 mM Tris–0.85% (wt/vol) NaCl–2.5% (wt/vol) Carnation skim milk powder (TNM; 16) for 2 h at 20°C with rocking. Appropriate nitrocellulose lanes were reacted with dilutions of rotavirus hyperimmune sera, postimmune sera, and mouse ascites fluids in TNM for 2 h at 20°C. After being washed in TNM, Tris-saline with 0.05% Nonidet P-40 and Tris-saline over a 30-min period with rocking, strips were immersed in TNM containing 0.1 μCi of ^{125}I-labeled protein A per lane (Amersham Corp., Arlington Heights, Ill.) for 30 min. After being washed as before, strips were air dried and exposed to Kodak XAR-5 film.

Rotavirus variants analysis. Variants of SA 11 rotavirus capable of producing plaques under an agarose overlay containing an excess of the SA 11-neutralizing monoclonal antibody A10/N3 were selected and kindly provided by S. Sonza, Division of Animal Health, Commonwealth Scientific and Industrial Research Organization, Parkville, Victoria, Australia. These variants were not neutralized by A10/N3 (see Table 5; S. Sonza, personal communication). The ability of Mab RV-3:1 and Mab RV-3:2 to neutralize these variants was assessed by FFN assay.

RESULTS

Production of monoclonal antibodies to rotavirus and characterization by EIA, FFN, and HI. Sufficient spleen cells were obtained from immunized mouse A (one booster injection) for two fusions (98 and 99) and from mouse B (two booster injections) for four fusions, although only two were performed (100 and 101); fusion 101 was done with twice the usual number (2×10^8) of spleen cells. The resulting numbers of hybrid cultures and screening results are shown in Table 1.

TABLE 2. Characteristics of neutralizing monoclonal antibodies to RV-3 (serotype 3) rotavirus

Antibody designation	Immunoglobulin class[a]	Titer against RV-3 as measured by[b]:		HI titer against antigen of strain[b]:		
		EIA	FFN	RRV-2	SA 11	K9
Mab RV-3:1	G2b	200,000	235,000	128,000	64,000	64,000
Mab RV-3:2	G2b	200,000	235,000	512,000	1,000	16,000
Mab RV-3:3	G2a	200,000	130,000	<10	10	<10

[a] Determined by immunodiffusion with antimouse class-specific and subclass-specific antisera (Miles Laboratories, Inc., Elkhart, Ind.).
[b] As ascites fluid. All lines were derived from separate clones.

TABLE 3. FFN titers of neutralizing monoclonal antibodies to RV-3 rotavirus against a panel of serotype 3 human and mammalian rotaviruses

Rotavirus[a]		Reciprocal neutralization titer of monoclonal antibody[b]		
Strain	Origin	RV-3:1	RV-3:2	RV-3:3
RV-3	Human, neonatal	235,000	235,000	130,000
RV-1	Human, neonatal	195,000	195,000	280
P	Human	330,000	425,000	195
YO	Human	170,000	2,350	<100
RRV-2	Simian	277,000	100,000	<100
SA 11	Simian	940,000	12,000	<100
K9	Canine	100,000	2,950	<100

[a] All virus stocks used in neutralization and EIA were determined to be of unique electropherotype by gel electrophoresis of viral RNA. The RNA of antigens was shown to co-electrophorese with viral RNA extracted from first-passage virus stocks. No extra RNA bands were present.
[b] Titer of mouse ascites fluid. Titers of the three monoclonal antibodies against human serotype 1 strains (Wa, KU, RV-4), human serotype 2 strains (DS-1, S2, RV-5), the human serotype 4 strain ST-3, and the UK bovine strain were all <100. Serotyping of RV-1, RV-3, and RV-4 was performed by FFN, using hyperimmune sera to Wa and SA 11 rotaviruses prepared as previously described (2) and Wa and SA 11 rotaviruses as controls. The serotype of RV-5 was determined by Dyall-Smith and Holmes (8). Serotyping of the remaining strains has been described previously (34).

As others have found (11, 13, 24, 27), the majority (95%) of rotavirus antibodies appeared to be cross-reactive between rotavirus strains. A further 1.6% of antibodies were specific for the serotype 3 rotaviruses tested (RV-3 and SA 11) but were nonneutralizing. The remaining 3.4% of antibodies neutralized RV-3. With increased spleen cell numbers, fusion 101 produced an increased yield of both EIA-positive and neutralizing antibody-producing hybrids. However, fewer than 50% of the latter hybrids survived beyond two subculture steps. The three surviving neutralizing clones fell into two groups: two appeared to be specific for serotype 3 viruses and inhibited HA; one reacted with homologous virus only and did not inhibit HA. These monoclones form the basis of the studies reported here. Their detailed characterization by EIA, FFN, and HI is

TABLE 4. EIA titers of neutralizing monoclonal antibodies to RV-3 against serotype 3 mammalian rotaviruses

Rotavirus[a]		Reciprocal EIA titer of monoclonal antibody:		
Strain[b]	Origin	RV-3:1[c]	RV-3:2[c]	RV-3:3[d]
RV-3	Human, neonatal	200,000	200,000	130,000
RV-1	Human, neonatal	200,000	200,000	800
P	Human	300,000	300,00	6,400
YO	Human	64,000	1,000	400
RRV-2	Simian	650,000	650,000	4,000
SA 11	Simian	650,000	8,000	3,200
K9	Canine	500,000	300,000	3,200

[a] See footnote *a* to Table 3.
[b] Titers of the three monoclonal antibodies against human serotype 1 strains (Wa, KU, RV-4), human serotype 2 strains (DS-1, S2, RV-5), the human serotype 4 strain ST-3, and the UK bovine strain were all ≤400. The serotyping of these rotaviruses was described previously. See footnote *b* to Table 3.
[c] As mouse ascites fluid.
[d] As affinity-purified mouse IgG prepared from ascites fluid, using a *Staphylococcus aureus* protein A-Sepharose 4B column (Pharmacia) as described previously (6) and adjusted to equivalent dilution to the mouse ascites starting material.

shown in Tables 2, 3, and 4. All were of the immunoglobulin (IgG) G class and reacted by EIA with homologous virus to a similar titer (1:200,000) (Table 2). However, Mab RV-3:3 showed a lower neutralization titer with RV-3 than did Mab RV-3:1 and Mab RV-3:2. Each of these latter two antibodies had a unique HI reaction pattern, indicating that although they are of identical IgG subclass, they are directed against different antigenic determinants of rotavirus.

For testing of the serotype specificity of the antibodies, a panel of mammalian rotaviruses of known serotype was assembled, and the FFN titers were determined for each monoclonal antibody (Table 3). The Mab RV-3:1 neutralized all seven serotype 3 strains to high reciprocal titer (100,000 to 940,000) and did not neutralize rotaviruses of serotype 1 (Wa, KU, RV-4), serotype 2 (DS-1, S2, RV-5), serotype 4 (ST-3), or the UK bovine strain. Similarly, Mab RV-3:2 neutralized only the seven serotype 3 viruses, but three of these were neutralized to lower titer than with Mab RV-3:1. These two monoclonal antibodies therefore appear to be serotype 3 specific but not identical. Interestingly, Mab RV-3:3 neutralized RV-3 (serotype 3) alone to high titer. Very much lower neutralization was evident with the other human strain RV-1 (serotype 3) and with rotavirus P, but none of the remaining serotype 3 strains, or strains of other serotypes, were neutralized by Mab RV-3:3. These three monoclonal antibodies also neutralized RRV-2 by plaque neutralization assay.

The EIA titers of the three monoclonal antibodies against the virus panel are shown in Table 4. Especially with Mab RV-3:1 and Mab RV-3:2, the EIA titers generally closely paralleled the neutralization titers for a given rotavirus. The one exception was the reaction of Mab RV-3:2 with K9 rotavirus, which was high titer by EIA but lower titer by FFN. Although the Mab RV-3:3 EIA titer was at least 20-fold greater to RV-3 (serotype 3) than to any other strain, EIA titers of 1:400 to 1:6,400 were obtained with other serotype 3 rotaviruses, even when affinity-purified monoclonal antibody was used. Hence, it appears that a low level of binding of Mab RV-3:3, not related to neutralization, may occur to serotype 3 rotaviruses.

The finding that Mab RV-3:3 neutralizes and reacts by EIA to a significant extent with RV-3 (serotype 3) but not with RV-1 (serotype 3) assumed importance when the history of these two isolates was considered. Strain RV-1 was isolated from a 5-day-old neonate in ward 21 at Royal Women's Hospital, Melbourne, in June 1977. Three months later, RV-3 was obtained from an infant of the same age in the same ward of that hospital. Viral RNA extracted from the two viruses was shown to coelectrophorese both before and after adaptation to cell culture (1). Rotaviral RNA extracted from RV-1 and RV-3 virus stocks used in FFN assays and EIA was found still to coelectrophorese (Fig. 1). These isolates have been described previously as electropherotype R (22), which was present over 6 years in the Royal Women's Hospital but not in the outside community.

The detection of a neutralization epitope on RV-3 not present on RV-1 rotavirus with Mab RV-3:3 suggests than an immunologically significant difference may exist between these two viruses. To test this, the ability of rabbit hyperimmune antiserum against double-shelled RV-3 virions (2) to neutralize RV-1 and RV-3 rotaviruses was assessed. The antiserum neutralized RV-3 to a titer of 1:134,000 and RV-1 to the significantly lower titer of 1:83,000 (Fig. 2). This experiment was repeated twice, and each point represents the mean of two replicates. Attempts to repeat this experiment with the original fecal material containing these viruses failed due to insufficient numbers of infectious rotavirus particles.

Polypeptide specificity of the RV-3 (serotype 3) neutralizing monoclonal antibodies. In Western blot analysis, none of the RV-3 neutralizing monoclonal antibodies bound to any reduced protein of RV-3 or SA 11 rotaviruses. However, Mab RV-3:3 bound to a single protein (molecular weight 62,000) of RV-3 when reacted with separated unreduced rotavirus proteins (Fig. 3). No binding of Mab RV-3:3 to unreduced SA 11 virus proteins was observed. The locations of the unreduced RV-3 virus proteins corresponding to gp34 of SA 11 (the neutralization antigen) and the major group antigen p42 (2, 14) are shown in lanes F and G of Fig. 3 with monoclonal antibodies C4/B3 and 4C8, which have been shown to immunoprecipitate gp34 and p42 of SA 11 virus, respectively (28). The Mab RV-3:3 therefore reacted with a protein unrelated to these rotaviral antigens. Comparison of the reduced RV-3 proteins detected by the use of anti-RV-3 serum (Fig. 3, lane A) with molecular weight standards allowed identification of the RV-3 virus proteins, by analogy with SA 11 rotavirus (2). Polypeptides 111, 98, 94, and 86 of RV-3 correspond to polypeptides 113, 96, 91, and 84 of SA 11. The molecular weights of p42 and gp34 did not vary between the two rotaviruses when reduced proteins were studied. The presence of the RV-3 virus protein p62, corresponding to p62 of SA 11 virus, the trypsin cleavage product of the outer capsid protein p84 (2) or VP3 (9), was notable. This protein, of the same molecular weight, was also detected by antisera in unreduced RV-3 and SA 11 virus

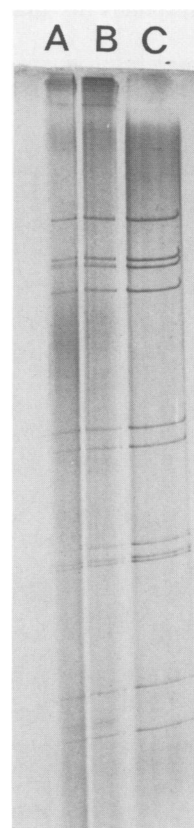

FIG. 1. Polyacrylamide gel electrophoresis of RV-1 and RV-3 rotavirus double-stranded RNA segments. Lanes: A, RV-1 RNA; B, RV-1 + RV-3 RNA; C, RV-3 RNA.

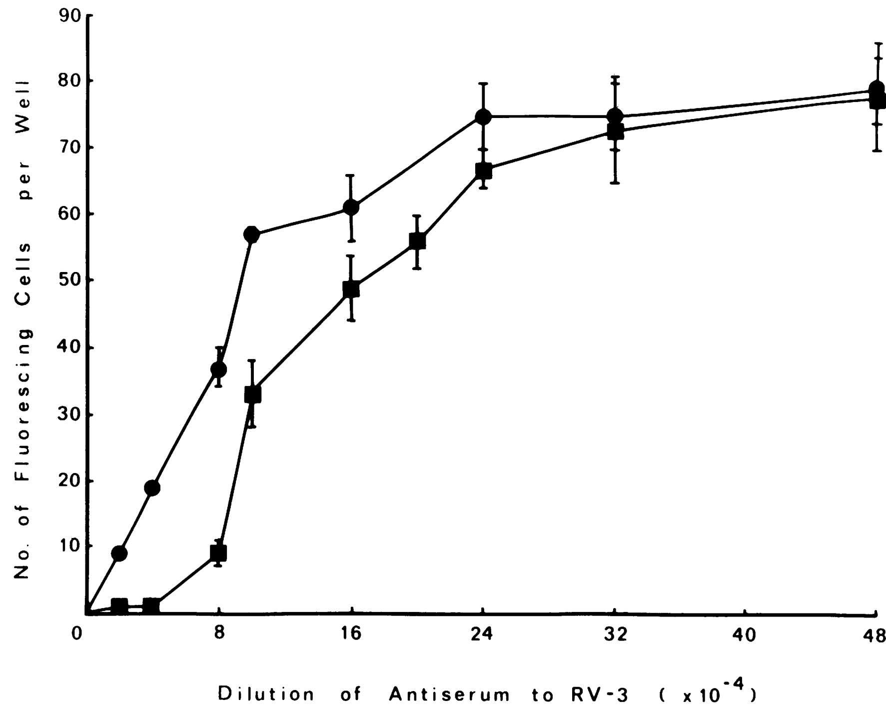

FIG. 2. FFN of RV-1 and RV-3 rotaviruses by rabbit hyperimmune serum to RV-3 rotavirus. Symbols: ●, RV-1 rotavirus; ■, RV-3 rotavirus.

preparations and corresponds to that detected by Mab RV-3:3.

The high-molecular-weight products detected by 4C8 are likely to represent p42 aggregates. The disappearance of

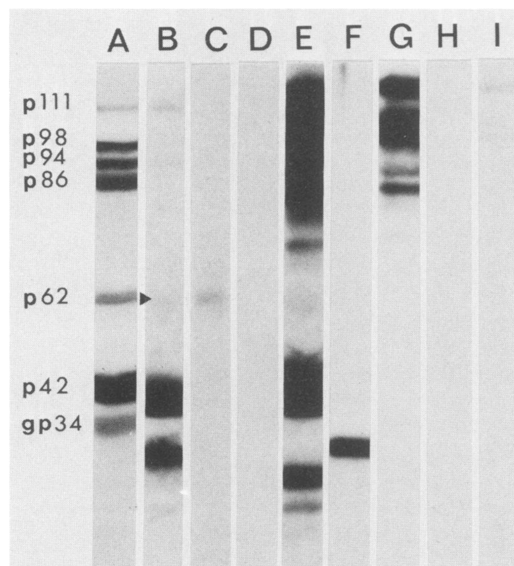

FIG. 3. Western blot analysis of binding of monoclonal antibodies and hyperimmune sera to RV-3 and SA 11 rotavirus proteins. Lanes: A, reduced RV-3 proteins + anti-RV-3; B, unreduced (UR) RV-3 proteins + anti-RV-3; C, UR RV-3 proteins + Mab RV-3:3; D, UR SA 11 proteins + Mab RV-3:3; E, UR SA 11 proteins + anti-SA 11; F, UR RV-3 proteins + C4/B3; G, UR RV-3 proteins + 4C8; H, UR RV-3 proteins + negative control mouse ascites fluid; lane I, UR SA 11 proteins + negative control mouse ascites fluid.

these aggregates in favor of the p42 band in the presence of 2-mercaptoethanol suggests that the polymeric forms of p42 are linked via disulfide bonds, as previously postulated (2).

Binding of Mab RV-3:1 and Mab RV-3:2 under reducing or nonreducing conditions to RV-3 or SA 11 rotaviruses in Western blots could not be demonstrated, so variant analysis was attempted. Variants of wild-type (wt) SA 11 rotavirus, selected with the SA 11-neutralizing monoclonal antibody A10/N3, which immunoprecipitates gp34 (28), were tested for their ability to be neutralized by Mab RV-3:1 and Mab RV-3:2. The FFN titers are shown in Table 5, together with titers to wt SA 11 rotavirus and A10/N3 (S. Sonza, personal communication). The Mab RV-3:1 showed a four-log reduction in neutralization titer with the SA 11 variants, which was comparable to that of A10/N3. Therefore, the change in gp34 elicited by A10/N3 has prevented binding of Mab RV-3:1 to the SA 11 rotavirus variants, and so Mab RV-3:1 reacts with an antigen on gp34. The Mab RV-3:2 did not show significantly different neutralization of the variants than wt SA 11 rotavirus, suggesting that if it binds to gp34, it is to a site unaffected by the change in gp34 elicited by A10/N3.

TABLE 5. FFN titers of RV-3-neutralizing and SA 11-neutralizing monoclonal antibodies with wt SA 11 and SA 11 variants

Virus	Reciprocal FFN titer of monoclonal antibody[a]		
	A10/N3	RV-3:1	RV-3:2
A10/314	100	400	8,000
A10/414	100	400	8,000
A10/514	100	200	8,000
SA 11 wt	$>10^6$	940,000	12,000

[a] As mouse ascites fluid.

DISCUSSION

Monoclonal antibodies directed to rotavirus antigens have been produced by a number of researchers (11, 13, 14, 24, 27, 28). However, we believe this to be the first description of the derivation of neutralizing monoclonal antibodies to a human rotavirus. One problem we encountered was that less than 4% of rotavirus antibody-secreting hybridomas were producing neutralizing antibodies to rotavirus. The instability (5) and inefficient production (25) of intact human rotavirus particles in cell culture may contribute to the low numbers of neutralizing monoclonal antibodies we derived to RV-3 (serotype 3) rotavirus, compared with neutralizing antibody numbers derived to animal viruses which grow well in cell culture, such as SA 11 (28) and RRV-2 (14). Our results indicated that repeated booster immunizations with RV-3 virus and doubling the number of spleen cells per fusion increased the number of neutralizing monoclonal antibodies detected.

The antibodies Mab RV-3:1 and Mab RV-3:2 proved to react specifically with serotype 3 rotaviruses, whether of human origin or from other mammals. Neither monoclonal antibody reacted with 10 cultivable rotaviruses of serotypes 1, 2, 4, and UK bovine. High titers to serotype 3 rotaviruses were shown by Mab RV-3:1, with good correlation between FFN and EIA results. This monoclonal antibody has now fulfilled its promise as a sensitive and specific serotype reagent, in an EIA developed for serotyping human rotaviruses. We have successfully serotyped a number of rotavirus strains with monoclonal antibodies to serotypes 2 and 3, and have produced serotype 4-specific monoclonal antibodies (unpublished data). Consistent with its serotype 3 specificity, Mab RV-3:1 was shown by FFN assay with variants of SA 11 rotavirus to bind to the serotype-specific component of RV-3 rotavirus equivalent to gp34 of SA 11 rotavirus. The binding site of Mab RV-3:1 on gp34 was related to that of the monoclonal antibody A10/N3 used to select the variants of SA 11; in competition studies this site is the apparently dominant neutralizing epitope of gp34 (28). This epitope is sensitive to reducing and denaturing agents, as it could not be detected by Mab RV-3:1 in Western blots even under nonreducing conditions. Radioimmunoprecipitation experiments with RV-3 or SA 11-infected cell lysates were also unsuccessful with Mab RV-3:1 and Mab RV-3:2 (S. Sonza and B. S. Coulson, unpublished data). This epitope is therefore likely to be exposed on the surface of the outer capsid (24) or to require the native protein conformation for activity.

Like Mab RV-3:1, Mab RV-3:2 showed serotype specificity and has proven effective in rotavirus EIA systems for serotyping human strains (unpublished data). Variant studies did not establish the binding site of Mab RV-3:2, although its ability to serotype suggests it is specific for a gp34 epitope not overlapping with the Mab RV-3:1 binding site. Competition studies and further variant analysis may decide this point.

Mab RV-3:3 appears to be directed at a tryptic product of the largest outer capsid protein (p86). However, this antibody only reacts by EIA with serotype 3 viruses. It is thus possible that antigens on p86 and gp34 tend to cosegregate. Further studies are needed to confirm this observation.

The finding that Mab RV-3:3 has substantial RV-3 (serotype 3) neutralizing activity and appears to be directed to the larger trypsin cleavage product of the largest outer capsid protein (p86) is of interest. Neutralizing monoclonal antibodies binding to the intact form of the larger outer capsid protein (p82) of RRV-2 (the viral hemagglutinin) have been elicited (14), but these antibodies also inhibited HA. As monoclonal antibodies directed to gp38, the serotype-related glycoprotein of RRV-2, also neutralized RRV-2 and inhibited its HA, it was not possible to conclude whether the neutralizing activity of the viral hemagglutinin-precipitating antibodies was related to epitopes on the viral hemagglutinin, or to steric hindrance of gp38 epitopes. Similarly, SA 11 virus-neutralizing monoclonal antibodies have been shown also to inhibit HA (28). Our studies with Mab RV-3:3, which did not inhibit HA by RRV-2, SA 11, or K9, suggest that p86 does contain at least one potential neutralization epitope. This site may be absent on the uncleaved protein, but it is present on the larger trypsin cleavage product (p62) of RV-3 rotavirus and so may be involved in determination of other functions of the protein such as host cell tropism and trypsin-enhanced plaque formation (17), rather than in viral HA.

The virtual absence of reaction of Mab RV-3:3 with RV-1 (serotype 3) rotavirus, isolated from the same ward of the same hospital as RV-3 (serotype 3) virus and of identical electropherotype, was surprising. The only difference previously noted between RV-1 and RV-3 rotaviruses is that RV-1 does not produce plaques in cell culture, whereas RV-3-infected cell cultures show thickened areas (1), similar to a bovine temperature-sensitive mutant found previously. Again, this suggests that the viral epitope detected by Mab RV-3:3 may relate to trypsin-enhanced plaque formation, and also that this site is subject to a form of antigenic drift. This latter conclusion is reinforced by the higher FFN titer of polyclonal anti-RV-3 hyperimmune serum found with RV-3 rotavirus than with RV-1 rotavirus, which suggests that an immunologically significant difference, not necessarily restricted to the difference detected with Mab RV-3:3, exists between RV-1 and RV-3 rotaviruses. This variation may prove analagous to the antigenic drift of the influenza virus hemagglutinin (35), or to the variation within poliovirus serotypes generated by selective pressure of antibody (7). The antigenicity (measured by virus neutralization) of bovine rotavirus strain Reims 18/77 was shown recently to alter drastically after 15 passages in cell culture in the presence of specific antibody (15). Virus adapted to culture without antibody showed unaltered neutralization kinetics. This may represent experimentally the endemicity of rotavirus strains such as RV-1 and RV-3 in nurseries of maternity hospitals in Melbourne, where for a number of years neonates receiving rotavirus antibody either in breast milk or across the placenta or both have been infected with mild or no symptoms (20). We plan further analysis of neonatal rotavirus strains, using Mab RV-3:3 and other monoclonal antibodies to investigate this phenomenon.

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