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Serological Analysis of the Subgroup Protein of Rotavirus, Using Monoclonal Antibodies

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Ten monoclones directed to the 42,000-dalton inner structural protein of rotavirus were analyzed. Eight monoclones reacted broadly with antigenic domains common to virtually all mammalian rotaviruses. Two monoclones had specificities similar or identical to previously characterized subgroup specificities. These subgroup monoclones were more efficient in detecting subgroup antigen than either hyperimmune or postinfection antisera. Using the subgroup monoclones, we determined that some animal as well as human rotavirus strains carry subgroup 2 specificity and that epizootic diarrhea of infant mice virus and turkey rotavirus are antigenically distinct from other mammalian rotavirus strains.

Rotaviruses cause severe infantile diarrheal illness in a wide variety of mammalian species including humans (14, 27). Early serological analysis of numerous rotavirus strains disclosed that all isolates studied shared common antigenic determinants (13, 29). However, later studies demonstrated that rotaviruses, when compared by neutralization assays, were antigenically quite distinct (2, 6, 7, 25, 29). In addition, it became apparent that for any single rotavirus strain, several different viral proteins possessed antigenic sites that were both distinct and readily detectable in serological assays (10, 12). Because of the antigenic complexity and diversity of rotavirus strains, a unified serological classification for these viruses has not yet been developed. However, two rotavirus serological specificities, serotype and subgroup, have been recently defined (12).

Rotavirus serotype refers to the specificity determined by viral neutralization (2, 7, 25, 29). At least in part, this specificity resides on the protein product of the eighth or ninth viral gene (10, 19; Greenberg et al., J. Gen. Virol., in press). This protein has been putatively identified as the 34,000-dalton (34K) to 37K surface glycoprotein (19). It appears that most animal species are naturally infected by serotypically distinct rotaviruses (26, 29). At least three distinct rotavirus serotypes have been isolated from humans (7, 25). Rotavirus subgroup, on the other hand, refers to the antigenic specificity associated with the protein product of the sixth gene (10, 12). The product of the sixth rotavirus gene is the 42K dalton major internal structural protein (18, 19, 23). This protein is present in large amounts on the virion and can readily be detected with a variety of antigen assay systems including complement fixation, immune adherence hemagglutination assay (IAHA), radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA). Two distinct nonoverlapping antigenic specificities now designated subgroup 1 and 2 have been identified on this protein during the study of a wide variety of rotaviruses (12). This antigenic polymorphism of the 42K dalton protein initially confused investigators since it was felt to reflect differences in neutralization specificity (31). Recent genetic studies have shown, however, that subgroup specificity segregates independently from neutralization specificity (10).

In an attempt to define more precisely a variety of rotavirus serological specificities, we have isolated monoclonal antibodies directed at several viral proteins. These monoclonal antibodies should also be helpful in understanding some basic aspects of protein function. In this study we have used 10 separate monoclonal antibodies directed at the sixth rotavirus gene product to define more accurately the antigenic nature of the subgroup protein.

MATERIALS AND METHODS

Viruses. Tissue culture-adapted human rotavirus (Wa strain) was triply plaque purified in African green monkey kidney cells, and a stock suspension was grown in MA104 cells (26). Human rotavirus strains DS-1, M, G, L, Fh, and DS-2 were derived from stools of gnotobiotic calves infected by the alimentary route (27). The St. Thomas-4 human rotavirus strain was isolated from an infant who was investigated as part of a well-studied outbreak of largely asymptomatic neo-

natal rotavirus infection in England (4). Human rotavirus strains HN8 and HN144 were isolated from ill children in Venezuela. An additional 25 human rotavirus strains (see Table 6) were also obtained from ill children in Venezuela. Human rotavirus strain KUN was isolated from a sick child in Japan (16). All the human rotavirus strains, other than Wa, were tested in the form of 1 to 10% suspensions of stool in veal infusion broth or phosphate-buffered saline. Bovine rotavirus (UK strain) was isolated by J. Bridges and G. Woode and provided by T. Flewett (3). NCDV bovine strain was provided by C. Mebus. Gottfried and OSU strains of porcine rotaviruses were provided by K. Theil and E. Bohl, Ohio Agricultural Research and Development Center. Canine rotavirus CU-1 strain was provided by Y. Hoshino (8). Simian rotavirus SA-11 strain was provided by H. Malherbe, and rhesus rotavirus strain 2 (MMU 18006) was provided by N. Schmidt (24). A turkey rotavirus strain isolated by S. McNulty was provided by W. Gary (20). The above rotaviruses were all passaged in MA104 cells before testing. Epizootic diarrhea of infant mice (EDIM) virus was kindly provided by M. Talty as a crude suspension of mouse intestines. The EDIM virus was serially passed 10 times in AGMK cells before testing (H. Greenberg and R. Wyatt unpublished data). Calf rotavirus strains C10, C486, and RS-1 were cultivated in BSC-1 cells and kindly provided by L. Babiuk. Bovine rotavirus strains B-14 and B682 were cultivated in MA104 cells and provided by G. Woode. A foal rotavirus strain was provided by T. Flewett in the form of a 20% fecal suspension. Porcine rotavirus EE (provided by E. Bohl) was cultivated in primary pig kidney. Human rotavirus Wa × bovine rotavirus UK reassortant viruses were isolated and characterized as previously described (4a, 6, 10).

Immunization. Two female 2-month-old BALB/c mice (no. 631 and 260) were immunized with the Wa strain of human rotavirus. One 2-month-old BALB/c mouse (no. 255) was immunized with the rhesus 2 strain of simian rotavirus. Rhesus rotavirus (RRV) was partially purified by high-speed centrifugation, fluorocarbon extraction, and rate zonal sedimentation through a 20 to 40% sucrose gradient (SW40 rotor, 35,000 rpm, 90 min). The fractions with peak viral hemagglutination titers were used for immunization. Wa virus was concentrated and partially purified for immunization by high-speed pelleting and fluorocarbon extraction. Mouse no. 260 was immunized with this partially purified preparation. The concentrated Wa virus was also treated with 0.05 M EDTA and then repelleted through 3 ml of 30% sucrose (SW40 rotor, 40,000 rpm, 3 h) for use in immunizing mouse no. 631. All three mice were initially immunized intraperitoneally with virus mixed with an equal volume of Freund complete adjuvant. After 1 to 2 months virus was administered intraperitoneally with an equal volume of incomplete Freund adjuvant. Animals were boosted one to three months after their second immunization by intravenous inoculation of virus suspended in phosphate-buffered saline (PBS). At 3 to 4 days after the booster inoculation the mice were sacrificed, spleens were removed, and spleen cells were separated by gently mincing with a surgical scalpel.

Fusion of spleen and NS-1 cells. The spleen cells were suspended in Dulbecco minimal essential medium (MEM) with gentamycin and pelleted (500 rpm, 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 cells-NS-1 cell mixture was again centrifuged (1,000 rpm, 5 min), and the supernatant was removed. The cell pellet was shaken gently and then placed in a 37°C water bath, and 1 ml of 35% polyethylene glycol 1000 in Dulbecco MEM was added over a 1-min period. After 6 min, 20 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 50 ml of standard medium (15% fetal calf serum, Dulbecco MEM with glutamine and gentamycin) and placed in a 150-cm^2 flask in 10% CO_2 overnight. The next day the cells were again pelleted and resuspended in 50 to 100 ml of hybridoma (HAT) medium as described by Kennett et al. (15). The cells were plated out in 24-well Costar plates (50 to 100 wells per spleen), and fresh HAT medium was added to wells on days 4, 6, 8, and 10. Wells with visible monoclones were tested by RIA when the medium became acid or the monoclones covered greater than 1/5 of the well bottom (or both). Monoclones of interest were cloned twice by limiting dilution with thymocyte feeder layers and then grown up in 20- to 40-ml pools for subsequent testing.

Solid-phase RIA. Polyvinyl chloride microtiter plates were precoated with a capture antibody consisting of hyperimmune goat antiserum (no. 930) to human rotavirus (D strain) diluted in PBS, pH 7.4 (30, 31). In the RIA this antibody reacted with all the other rotaviruses used in this study. After an overnight incubation at room temperature, plates were washed three times with PBS, and 50 μl of test rotavirus antigen in the form of crude cell culture harvest or fecal suspension was then added to the microtiter wells and allowed to bind overnight at room temperature. Different passage levels of the various cultivatable rotaviruses gave similar results in the solid-phase assay. Plates were then washed four times with PBS, and test monoclone fluid was added (50 μl per well). Plates were then incubated for 1 to 2 h at 37°C. Plates were again washed four times, and 50 μl of ^{125}I-labeled goat anti-mouse immunoglobulin G, Fc specific (Cappel Laboratories, 100,000 cpm per well), was added to the microtiter plate. The antiserum was iodinated by a modification of the Hunter-Greenwood reaction (21). After an additional 1-h incubation at 37°C plates were washed four times, and individual wells were cut out 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 (approximately 100 cpm). Wells with 3.5 times as many counts bound as the negative controls were considered positive (P/N $\geqq$ 3.5). This ratio (P/N) was selected because preliminary testing indicated that using it allowed the assay to be both reproducible and sensitive. Similar criteria were used for choosing ratios of 0.5 and 3 in the subgrouping assays (see Table 5). In initial screening tests for antibody production by monoclones, plates were coated with homologous immunizing rotavirus (RRV or Wa). For studies of differential binding plates were coated with a variety of rotaviruses (Table 1). All assays were run in duplicate, and all tests were repeated at least once.

IAHA and ELISA subgroup assays. The IAHA and ELISA subgroup assays were performed as previously described (12).

Electropherotype. Electropherotype analysis of viral RNA was performed on the 25 human rotavirus strains from Venezuela (see Table 6) as well as most of the other rotavirus strains studied (Table 1). In all cases rotavirus was partially purified from stool or tissue culture by fluorocarbon extraction and high-speed pelleting through 30% sucrose. Viral RNA was purified by phenol-chloroform extraction and ethanol precipitation (9, 11). Polyacrylamide gel electrophoresis of virion RNA was performed with the Laemmli buffer system as previously described (10).

Preparation of radiolabeled infected cell lysates. Proteins were labeled by a modification of the method of Lee et al. (17). MA104 monolayers were infected with virus (Wa or RRV) at multiplicities of infection ranging from 1 to 10. Virus was initially treated with trypsin (5 μg/ml, 1 h). After a 1-h absorption period monolayers were washed and refed with Eagle MEM with glutamine and antibiotics. At 5 to 8 h postinfection the cells were again washed and refed with Eagle MEM without methionine. After 45 min the cells were again washed once and refed with Eagle MEM without methionine, but with 150 mM NaCl added (22). After 15 min 50 μCi of [^{35}S]methionine (Amersham/Searle; 800 to 1,200 Ci/mmol) per ml was added, and incubation was continued for another hour. Monolayers were then washed three times with PBS, 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 and 1% Triton X-100 (100 μl of buffer per 4×10^5 cells) followed by a buffer containing 10 mM Tris-hydrochloride (pH 7.8), 1 mm phenylmethylsulfonyl fluoride, and 1% Triton X-100 (400 μl of buffer per 4.0×10^5 cells). The rotavirus-infected cell lysate was then spun for 2 h at 40,000 rpm (SW40 rotor) and stored for use at −70°C.

Immunoprecipitation. Lysate (50 μl) was added to 50 μl of hybridoma culture fluid. The mixture was incubated at 37°C for 1 h and then at 4°C overnight; 50 μl of 10% *Staphylococcus aureus* cells (Calbiochem Pansorbin) was then added, and the mixture was incubated for 15 min. The bacteria were then pelleted (10,000 rpm in a 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 deoxycolate, 0.1% Nonidet P40, 0.1% Triton X-100, 0.001 M phenylmethylsulfonyl fluoride. Bound labeled proteins were removed from the *S. aureus* 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 cells were again pelleted by centrifugation, and the supernatants were applied to 12% sodium dodecyl sulfate-polyacrylamide gels as previously described (5, 17). Gels were dried and autoradiographed as previously described (4a).

Neutralization assay. Serotype designations were determined by 60% plaque reduction assay as previously described (25).

RESULTS

Our intent in these studies was to isolate monoclonal antibodies directed at the various surface and internal structural viral proteins of human and animal rotaviruses. However, by using a solid-phase RIA as the primary screening technique, the majority (70%) of isolated hybridomas was observed to be directed at the 42K dalton major inner structural protein of the rotavirus even though many of the hybridomas were derived from two animals (no. 255 and 260) immunized with complete or complete and incomplete virions. We selected 10 of the monoclones directed at the 42K protein for further study. Six of the monoclonal antibodies (mice no. 631 and 260) were derived from mice immunized with Wa human rotavirus (Table 1). Mouse no. 631 was immunized with incomplete single-shelled viral particles to select preferentially for monoclones to internal viral proteins. Four of the monoclones (mouse no. 255) were isolated from a mouse immunized with rhesus rotavirus (Table 1). Each of the 10 monoclonal antibodies immunoprecipitated the 42K viral protein from the immunizing virus. In Fig. 1 the six monoclonal antibodies derived from the Wa-immunized mice are shown precipitating the 42K inner protein of Wa virus. The RRV-derived monoclonal antibodies immunoprecipitated the same 42K protein from RRV-infected cell lysates (data not shown). None of the monoclonal antibodies had detectable neutralizing activity against Wa or RRV when hybridoma supernatants were tested at a 1:10 dilution.

We next investigated the antigenic specificity of the 10 monoclonal antibodies that precipitated the 42K protein by measuring monoclonal binding in the RIA to a series of 11 separate rotaviruses (Table 1). The series includes two human strains (DS-1 and Wa) that are distinct serotypes and in addition belong to different subgroups (12), several mammalian rotavirus strains, and one avian strain (Table 1). All monoclone culture supernatants were tested at a single dilution (1:2). Of interest was the finding that all but two (631/9 and 255/60) of the 10 monoclonal antibodies reacted with an antigenic site present on most rotaviruses, with the exception of the one avian strain. The suspension of avian rotavirus used in these assays had an infectivity titer ($10^{6.5}$ PFU/ml) similar to that of the other cultivatable rotaviruses studied. Monoclones 631/7 and 260/12 reacted with all of the mammalian rotavirus strains, except for OSU porcine and NCDV bovine strains, respectively. Only one monoclonal antibody (255/34) reacted with the turkey rotavirus. Thus immunization with Wa or RRV resulted in the production of monoclones directed against the major inner rotavirus protein with strong heterologous reactivity (Table 1). Although most of the monoclonal antibodies bound to common antigenic sites on the 42K protein of many rotaviruses, two hybridomas

TABLE 1. Binding of 10 monoclones directed at the 42K inner rotavirus protein to various human and animal rotavirus strains as measured by solid-phase RIA

Monoclone designation[a]	P/N ratio[b] of rotavirus strain:										
	Human DS-1	Human Wa	Bovine UK	Simian RRV2	Canine CU1	Bovine NCDV	Porcine OSU	Mouse EDIM	Simian SA11	Avian turkey	Porcine Gottfried
631/9	3.1	**39**	1.6	1.9	1.4	1.7	0.5	4	0.6	0.5	12
631/7	24	**29**	30	24	30	30	0.9	24	22	0.3	24
631/16	27	**22**	20	30	22	19	22	21	27	0.8	24
631/24	25	**19**	25	25	21	21	27	26	27	1.0	20
260/12	8.6	**9.9**	12	9.4	18	1.6	15	8.2	9.1	0.6	7.3
260/19	13	**33**	41	31	42	32	34	14	23	0.6	4.8
255/10	6.2	7.9	17	**16**	13	16	5.9	11	7.1	0.4	6.1
255/18	24	24	30	**32**	26	29	26	20	27	0.7	19
255/34	3.6	4.2	4.7	**11**	14	5.3	6	12	12	4.8	3.6
255/60	26	1.9	40	**31**	31	33	32	1.6	24	0.7	1.1

[a] Monoclones 631/9, 631/7, 631/16, 631/24, 260/12, and 260/19 were derived from mice immunized with Wa virus. Monoclones 225/10, 255/18, 255/34, and 255/60 were derived from a mouse immunized with rhesus rotavirus. All monoclonal antibodies were of the immunoglobulin G1 subclass, except for 255/10, which has not been classified.

[b] The P/N ratio is the counts per minute of indicated monoclone bound to microtiter wells coated with indicated virus divided by the counts per minute of the indicated monoclone bound to microtiter well coated with uninfected tissue culture. Homologus P/N ratios are printed in boldface type. A P/N ratio of ≥3.5 was considered significant.

appeared to react more selectively. In the initial screening monoclone 631/9 reacted strongly with only the human rotavirus Wa strain and the Gottfried strain of porcine rotavirus. On the other hand, monoclone 255/60 reacted with the DS-1 human rotavirus, the simian rotaviruses, the bovine rotaviruses, the canine rotavirus, and the OSU porcine rotavirus (Table 1). Since the reactivity defined by these two monoclonal antibodies was similar to the previously described subgroup specificity (12), we studied these two hybridomas in greater detail.

Preliminary screening (Table 1) disclosed that monoclone 631/9 reacted with Wa virus (a subgroup 2 virus), but not with UK virus (a subgroup 1 virus), whereas monoclone 255/60 react-

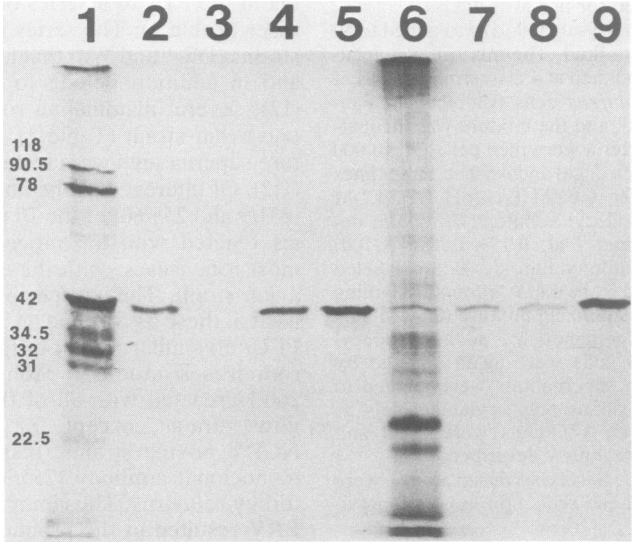

FIG. 1. Immunoprecipitation of [^{35}S]methionine-labeled, Wa rotavirus-infected cell lysate by indicated monoclone. See the text for the procedure. Lanes: 1, goat hyperimmune anti-human rotavirus D strain; 2, monoclone 631/9; 3, NS-1 supernatant; 4, monoclone 260/19; 5, monoclone 260/19; 6, Wa lysate alone; 7, monoclone 631/16; 8, monoclone 631/24; 9, monoclone 631/7. The molecular weights of the indicated rotavirus proteins are shown at the left of the figure (5).

TABLE 2. Binding of subgroup specific monoclones 631/9 and 255/60 to various rotavirus reassortants derived from coinfection with Wa and UK rotaviruses

Monoclone designation	P/N ratio of monoclone bound to indicated rotavirus reassortant						
	1-1[a]	18-1[b]	11-1[c]	9-1[d]	36-1[e]	Wa parent	UK parent
631/9	22	0.9	15	1.1	12	30	1.4
255/60	1.4	20	1.2	20	1.4	0.7	29

[a] Reassortant genes 1, 3, 4, 5, 7, 8, and 10 were derived from the UK parent.
[b] Reassortant genes 1, 2, 4, 5, 6, 7, and 10 were derived from the UK parent.
[c] Reassortant genes 4, 7, 9, and 10 were derived from the UK parent.
[d] Reassortant genes 1, 2, 4, 5, 6, 7, 9, and 10 were derived from the UK parent.
[e] Reassortant genes 1, 2, 4, 5, and 9 were derived from the UK parent.

ed with UK virus, but not with Wa virus. We took advantage of this selective specificity by measuring the binding of the two monoclones to a series of reassortant rotaviruses derived from cells coinfected with Wa and UK rotavirus (Table 2). The genotypes of these reassortants had previously been determined (4a, 10). Monoclone 631/9 only bound to reassortants whose sixth gene was derived from Wa virus, whereas monoclone 255/60 reacted exclusively with reassortants whose sixth gene was derived from the UK parent. Thus, by a genetic analysis these monoclones could be shown to react exclusively with the sixth gene product, the subgroup protein. In addition, they appeared to have specificities similar to the subgroup specificities previously defined by both absorbed hyperimmune and postinfection antisera (12).

The specificity of the two monoclonal antibodies was also assessed by titering them against single dilutions of two subgroup 1 and two subgroup 2 viruses (Table 3). Reactivity was highly specific. In addition the four viruses, two belonging to subgroup 2 and two belonging to subgroup 1, were titered in solid-phase RIA against single dilutions of the two monoclones (Table 4). Again, the reactivity appeared to be specific.

Twenty-nine rotavirus strains (Table 5) of human and animal origin were used to compare the specificity of monoclones 631/9 and 255/60 to our previously defined conventional subgroup specificity assay. The RIA binding activity for the two monoclonal antibodies was combined into a ratio. The antigen binding (counts per minute in the RIA) of monoclone 631/9 was divided by antigen binding of monoclone 255/60. Ratios greater than 3 were considered indicative of subgroup 2 specificity, and ratios less than 0.5 were considered indicative of subgroup 1 specificity. The two subgrouping assays (Table 5) were in close agreement. As previously reported human rotavirus strains possess either subgroup 1 or 2 specificity (9, 12). It is noteworthy that previously, all animal rotavirus studied, with the exception of EDIM, had been characterized as subgroup 1 viruses (12). However in this study, two previously untested strains of equine and porcine origin (Gottfried and Foal rotavirus, Table 5) were identified as subgroup 2 viruses. When one of these strains was retested in ELISA or IAHA with our conventional antisera, the subgroup 2 classification was verified (Table 5).

Finally, we compared the sensitivity of the two subgrouping monoclonal antibodies to our previously described conventional subgroup reagents. Twenty-five human rotavirus strains from Venezuelan children with diarrhea were

TABLE 3. Antibody titer by RIA of monoclones 255/60 and 631/9 against subgroup 1 and 2 rotaviruses[a]

Monoclone designation	Reciprocal titer of monoclone against indicated rotavirus (origin and subgroup)			
	Wa (human 2)	D (human 2)	DS-1 (human 1)	Rhesus 2 (simian 1)
631/9	≥5,120	≥5,120	5	5
255/60	<5	5	≥5,120	5,120

[a] Viruses were added to microtiter wells at a 1:2 dilution. A P/N ratio of ≥3.5 compared with the negative control was considered significant.

TABLE 4. Antigen titer by RIA of subgroup 1 and 2 viruses determined with a single dilution of monoclones 631/9 and 255/60

Virus	Reciprocal titer of subgroup 1 or 2 viruses with indicated monoclone[a]	
	631/9	265/60
Human rotavirus WA (2)[b]	80	<10
Human rotavirus D (2)	160	<10
Rhesus rotavirus 2 (1)	<10	2,560
Human rotavirus DS-1 (1)	<10	2,560

[a] Both monoclones were used at a 1:5 dilution. A P/N ratio of ≥3.5 compared with the negative control was considered significant.
[b] The numbers within parentheses indicate subgroup specificity as determined by ELISA or IAHA (or both).

TABLE 5. Subgroup specificity assay of animal and human rotavirus strains with monoclones 631-9 and 255-60

Specimen designation	Origin	Subgroup ratio[a]	Subgroup specificity[b]	Similar or identical to indicated strain by plaque reduction
Wa	Human	8.4	2	Wa
D	Human	8.6	2	Wa
M	Human	5.3	2	RRV
G	Human	8.1	2	Wa
L	Human	7.2	2	Wa
Fh	Human	8.9	2	Wa
St. Thomas-4	Human	4.6	2	Neither Wa nor DS-I
DS-2	Human	14	2	—[c]
DS-1	Human	0.03	1	DS-1
HN-8	Human	0.2	1	—
NH-144	Human	0.15	1	—
KUN	Human	0.33	1	DS-1
RRV-2	Simian (Rhesus monkey)	0.15	1	RRV
SA-11	Simian (Vervet monkey)	0.22	1	RRV
Feline	Feline	0.26	1	RRV
Canine	Canine	0.19	1	RRV
NCDV	Bovine	0.16	1	NCDV
UK	Bovine	0.14	1	NCDV
C10	Bovine	0.3	1	—
C486	Bovine	0.08	1	—
RS1	Bovine	0.06	1	—
B-14	Bovine	0.08	1	—
B682	Bovine	0.25	1	—
OSU	Porcine	0.19	1	OSU
EE	Porcine	0.07	1	—
Gottfried	Porcine	16.0	2[d]	—
Foal	Equine	4.0	—	—
EDIM	Murine	2.5	Neither 1 nor 2	—
Turkey	Avian	0.7	—	—

[a] Binding in RIA of monoclone 631/9 divided by that of monoclone 225/60. A ratio of >3 is indicative of subgroup 2 specificity; a ratio of <0.5 is indicative of subgroup 1 specificity.
[b] Determined by conventional ELISA or IAHA (or both).
[c] —, Serotype or subgroup unknown or not tested.
[d] Determined after results of RIA were known.

analyzed by conventional subgroup assays, including ELISA and IAHA, with both hyperimmune and infection antisera. In addition, virion RNA from these Venezuelan specimens was purified and studied by gel electrophoresis. Viruses with RNAs having the so-called "short" pattern were classified as subgroup 1 viruses (9). When conventional subgroup antigen assays (IAHA and ELISA with calf infection sera) were used, assignment of subgroup could be made for only 14 strains (Table 6). Eleven strains could not be characterized because of insufficient antigen in the fecal specimen. These 11 strains required characterization by RNA gel electrophoresis. Twenty-five strains were classified by using the subgroup monoclones in an RIA test. This assay was in complete agreement with the subgroup designation determined by conventional subgroup assays including ELISA, IAHA, and electropherotype. Consistent with our previous finding, all six viruses with a short RNA pattern were subgroup 1 (9).

DISCUSSION

Monoclonal technology provides unique opportunities for developing highly specific reagents for classifying and comparing various viral strains. As part of a project to use monoclonal antibodies to investigate rotavirus protein structure and function, we have isolated and characterized 10 separate monoclonal antibodies directed at the major viral internal protein. Monoclones to this protein were the predominant species detected and recovered when hybridomas were screened by a solid-phase RIA. This prevalence probably reflects both the abundance of the 42K protein in the virion and its strong antigenicity.

The specificity of the 10 monoclonal antibodies for the 42K protein was documented in two ways. First, all of these monoclones specifically precipitated the 42K protein from infected cell lysates. In addition, the specificity of two of the monoclones (631/9 and 255/60), which bound

TABLE 6. Correlation of subgroup specificity of selected HRV strains as detected with monoclonal antibodies or previously described subgrouping analysis (IAHA, ELISA, or electropherotype)

Previous subgroup designation[a]	Subgroup designation with indicated monoclone	
	1 (255/60)	2 (631/9)
1	6	0
2	0	19

[a] Determined by IAHA, ELISA, or electropherotype (or a combination); 11 of 25 strains were classified on the basis of electropherotype alone.

selectively to only certain rotaviruses, was determined by genetic analysis. These two monoclones reacted with a series of rotavirus reassortants in a pattern indicating that they were directed against the product of the sixth viral gene (Table 2). Several in vitro translation studies have shown that the protein product of the sixth RNA segment is the 42K major inner core protein (18, 19, 23).

Many serological studies have demonstrated the high degree of antigenic relatedness among rotavirus strains, even those from different species (13). Eight of the 10 monoclones directed at the 42K protein recognized antigenically similar regions in a wide variety of rotavirus strains. Such monoclonal antibodies, which were derived from mice immunized with either Wa or RRV, bound equally well to the 10 mammalian rotavirus strains studied. Interestingly, only the avian rotavirus strain appeared to be rather distinct when studied with these hybridomas. The cross-reactivity detected in hyperimmune rotavirus antisera is probably due in large part to the shared antigenic regions on the 42K inner protein.

Two monoclones reacted with a region on the 42K protein that exhibited greater specificity. Monoclones 631/9 and 255/60 demonstrated reactivity identical to our previously defined subgroup specificity (12). Monoclone 631/9 reacted with Wa rotavirus and with all other human rotavirus strains tested that had a "long" RNA pattern. At least two, and probably three, human serotypes were recognized by this monoclonal antibody (Table 5). Of note, two rotavirus strains of animal origin, a porcine and an equine strain, reacted with this monoclone in the solid-phase RIA. Previously, subgroup 2 specificity had only been detected on human rotaviruses (12). Whether the subgroup 2 porcine and equine strains represent distinct animal rotaviruses, whether they were originally of human origin but have moved into an animal species, or indeed whether these viruses represent reassortants with human rotavirus, remains to be determined. In any case, among rotaviruses thus far studied subgroup 2 specificity is clearly most commonly found on human strains. Monoclone 255/60 reacted with all the mammalian animal rotavirus strains studied, with the exception of two subgroup 2 porcine and equine strains and EDIM. Monoclone 255/60 also reacted with all human rotavirus strains with short RNA patterns. Of the mammalian strains studied only EDIM appeared to fall outside the two subgroup specificities.

The present studies were not designed specifically to characterize the number of distinct epitopes on the 42K rotavirus protein. Blocking studies with radiolabeled and nonradiolabeled monoclones will be required to make that determination. However, the data do support the proposition that at least two distinct domains exist on the 42K rotavirus protein. One domain, to which 8 of the 10 monoclones were directed, is characterized by an antigenic region that is shared among all mammalian rotaviruses. The other domain, which is antigenically polymorphic, can have at least two specificities. It is this region that is detected in subgrouping assays. Of interest is the finding that when gnotobiotic calves undergo rotavirus infection the response to the sixth gene product is primarily to the subgroup region of the 42K protein. For this reason postinfection gnotobiotic calf sera can be used in subgroup assays (12). It is unknown whether humans also preferentially respond to the subgroup region of the 42K protein during primary infection. On the other hand, hyperimmune sera usually contain antibodies directed at both domains and hence must be absorbed to be used effectively in subgroup assays (12).

The subgroup monoclones offer several advantages for subgroup testing when compared with either the absorbed hyperimmune antiserum or the gnotobiotic calf postinfection antiserum (12). They are available in unlimited amounts and have uniform characteristics. Most importantly the monoclones are more sensitive than the conventional reagents. Of 25 human rotavirus strains tested by conventional subgroup assay, only 14 could be classified. With the two subgroup monoclones all 25 rotaviruses could be subgrouped (Table 6). The availability of these monoclones will allow us, for the first time, to classify rapidly and accurately the majority of field isolates without resorting to concentration or purification. We have adopted these two monoclones for use in the ELISA by a system identical to the previously published ELISA subgroup assay, except that an anti-mouse immunoglobulin G conjugate is used (30, 31). The ELISA assay is sensitive, specific, and reliable. These monoclonal antibodies did not react efficiently in the IAHA.

The epidemiological significance of subgroup classification can now be easily investigated. There are potentially, however, several very practical uses for the subgroup monoclones. Environmental rotavirus isolates such as might be found in drinking water or reservoirs can be subgrouped as a preliminary step in investigation. Isolates that are subgroup 2 would seem to have a high probability of being human in origin and could be further studied. The search for evidence of animal rotavirus transmission to humans might be facilitated with these subgroup reagents. High-risk human populations, such as farm children or rural households, could be screened with the subgroup reagents. Subgroup 1 strains, which should be in the distinct minority, would then be studied by RNA analysis. Subgroup 1 isolates with a so-called long pattern would have a high likelihood of being an animal rotavirus (serotype) and could be further studied by in vitro cultivation and neutralization.

The 10 monoclonal antibodies directed at the 42K inner viral protein should provide useful probes for studying viral assembly and structure. None of the 10 monoclones studied had neutralizing activity. Bastardo and co-workers have reported that hyperimmune sera made in animals immunized with the 42K protein did contain low levels of neutralizing activity (1). Further analysis will be needed to see whether the 42K protein is indeed present on the virion surface and, if so, whether the antigenic region on the viral surface represents a distinct domain.

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