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Reactivities of Serotyping Monoclonal Antibodies with Culture-Adapted Human Rotaviruses

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Rotaviruses collected in Bangladesh during 1985 to 1986 were culture adapted and used in a comparative serotyping study with three groups of monoclonal antibodies, all of which reacted with the major neutralization protein (VP7) of serotype 1, 2, 3, or 4. The goals were to determine which monoclonal antibodies most accurately predicted the serotype and why large variations in serotyping efficiencies have occurred with these monoclonal antibodies in previous studies. The 143 rotavirus isolates used in this study belonged to 69 different electropherotypes; and 44, 23, 21, and 55 isolates were identified as serotype 1 through 4, respectively, by neutralization with serotype-specific hyperimmune antisera. Serotyping specificity by enzyme-linked immunosorbent assay with monoclonal antibodies was 100% consistent with results found by neutralization with polyclonal antisera, but large differences were observed in the sensitivities of the different monoclonal antibodies. Monoclonal antibodies 5E8 (serotype 1), 1C10 (serotype 2), 159 (serotype 3), RV3:1 (serotype 3), ST-3:1 (serotype 4), and ST-2G7 (serotype 4) reacted with all the isolates of the corresponding serotype for which there were sufficient infectious particles. Monoclonal antibody 2F1 (serotype 2) was much less sensitive and reacted with only five serotype 2 isolates, but these were among those with the highest titers. Monoclonal antibodies RV4:2 (serotype 1), KU6BG (serotype 1), RV5:3 (serotype 2), and S2-2G10 (serotype 2), on the other hand, failed to react with between one and three isolates of the corresponding serotypes which had high titers, apparently because of epitope changes in these isolates. Effects of epitope variation were, however, most apparent with monoclonal antibodies 2C9 (serotype 1) and YO-1E2 (serotype 3), which reacted with one and no isolates of the corresponding serotypes, respectively. Cross-neutralization of escape mutants indicated that the serotype 1 monoclonal antibodies 5E8, 2C9, and RV4:2 reacted with different but probably overlapping epitopes, as did serotype 2 monoclonal antibodies 2F1, 1C10, and RV5:3, findings that were consistent with the enzyme-linked immunosorbent assay data. Because of epitope variations between rotavirus strains, serotyping with several monoclonal antibodies directed at different epitopes may increase the sensitivity of the method.

Rotaviruses are the principal etiologic agents responsible for severe diarrhea in infants and young children, and vaccines to protect against rotavirus disease are in various stages of development. Although immunity to rotavirus disease is poorly understood, it has been suggested that protection following vaccination may be serotype specific (12, 13, 19, 27). This is a key element in rotavirus vaccine development and needs to be verified by analysis of the serotype specificity of protection in rotavirus vaccine trials.

Rotavirus serotypes have been defined primarily by immune responses to antigenic determinants on the outer capsid VP7 protein. Until recently, serotyping of human rotaviruses was performed by neutralization of culture-adapted isolates by using serotype-specific polyclonal antibodies, a very labor-intensive procedure. Within the past few years, however, several laboratories have produced neutralizing monoclonal antibodies that recognize rotaviruses belonging to specific serotypes (2, 9, 20, 28, 30). Serotype-specific monoclonal antibodies to VP7 have been used to determine the serotypes of human rotaviruses directly in stool specimens by enzyme-linked immunosorbent assays (ELISAs) (2, 5, 9, 25, 30, 34). The success rate of serotyping with monoclonal antibodies has been extremely variable (1, 3, 6, 7, 10, 14–16, 24, 25, 31, 33). Possible causes of this variability include, but are not limited to, breakdown of intact double-shelled rotavirus particles, the absence of recognizable epitopes by particular monoclonal antibodies because of strain variations, and the presence of inhibitory factors in the stool preparations. Each of these causes requires a different solution to maximize the efficiency of serotyping with monoclonal antibodies.

A study on the epidemiology of rotavirus disease in Bangladesh was recently completed during which 335 strains possessing 11 distinct double-stranded RNA segments were successfully adapted to grow in cell culture (37). These strains, which belonged to 69 clearly distinct electropherotypes, were subsequently serotyped by a focus reduction neutralization assay by using polyclonal antisera made in guinea pigs hyperimmunized with representatives of rotavirus serotypes 1 to 4. All 335 isolates were serotypically related to one of these four serotypes, and cross neutraliza-

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tion by antisera against two or more of these serotypes was observed in only eight isolates. A relatively equal distribution of these isolates was found within the four serotypes; i.e., 80, 48, 119, and 88 isolates were identified as serotypes 1 through 4, respectively.

These culture-adapted rotavirus isolates from a geographically defined population under comprehensive systematic epidemiologic surveillance are unique reagents with which to determine the basis of serotyping variabilities that have been reported by using monoclonal antibodies. Potential inhibition of the serotyping assay because of stool components could be eliminated because each isolate was grown in cell culture. Furthermore, the relationship between virus titer and sensitivity of the assay could be explored because the number of infectious particles present in each preparation was determined. Finally, the relative efficiency of serotyping numerous isolates belonging to each of the four major rotavirus serotypes by different monoclonal antibodies could be determined because the serotypes of all isolates were previously identified by neutralization with polyclonal antibody preparations.

MATERIALS AND METHODS

Viruses. Laboratory strains representative of each of the six established human rotavirus serotypes were grown in MA104 cells, and aliquots of each virus preparation were stored at −70°C. The following laboratory strains were used: Wa (serotype 1), DS-1 (serotype 2), P (serotype 3), ST-3 (serotype 4, subtype 4A), VA70 (serotype 4, subtype 4B), 69M (serotype 8), and WI61 (serotype 9). Field strains of human rotaviruses obtained from stool specimens of subjects with diarrheal disease in the Matlab area of Bangladesh during 1985 to 1986 were culture adapted by two passages in primary African green monkey kidney cells as described previously (35) and grown for an additional four passages in MA104 cells before being stored in aliquots at −70°C. The titer of infectious rotaviruses in all preparations was determined by a fluorescence focus assay (4) and varied between 3×10^3 and 3×10^7 focus forming units (FFU) per ml.

Selection of field isolates for comparative serotyping. A total of 454 episodes of rotavirus-associated diarrhea were detected in the study population, and 381 (84%) strains were successfully adapted to grow in cell culture (37). Comparative electrophoretic analysis of rotaviral genomic RNA segments extracted from the original stool specimen and the fifth cell culture passage revealed that 46 strains had additional segments, possibly because of dual rotavirus infections. The 335 isolates that were electrophoretically "pure" were serotyped by a neutralization assay with polyclonal antibody made in guinea pigs hyperimmunized (21) with representatives of serotypes 1 to 4, i.e., strains Wa, DS-1, P, and ST-3, respectively. All were identified as serotype 1, 2, 3, or 4. Eight isolates were weakly neutralized by antisera to more than one serotype, but the dominant serotype was evident in every case based on differences in titers. Within these 335 culture-adapted isolates, there were 69 clearly distinguishable electropherotypes. Thirty-two of these were represented by a single isolate, while, at the other extreme, one electropherotype had 79 representatives, all belonging to serotype 3. At least one representative of each electropherotype was included in this study and, if an electropherotype contained more than one representative, at least two isolates of that electropherotype were included. When unusual results were found for any particular isolate, additional representatives of that electropherotype were examined. The maximum number of representatives of any electropherotype was five. All eight isolates neutralized by more than one polyclonal antibody preparation were included. The total number of culture-adapted isolates examined was 143.

Monoclonal antibodies used for serotyping. All 143 rotavirus isolates used in this study were serotyped with monoclonal antibodies from three different sources, each of which was directed against the VP7 protein. Group 1 was from H. Greenberg and included serotype 1 (2C9, 5E8), serotype 2 (2F1, 1C10), and serotype 3 (159) monoclonal antibodies. Group 2 was from B. Coulson and included one monoclonal antibody each to serotype 1 (RV4:2), serotype 2 (RV5:3), serotype 3 (RV3:1), and serotype 4 (ST-3:1). Group 3 was prepared by K. Taniguchi et al. (30) and included strains KU6BG (serotype 1), S2-2G10 (serotype 2), YO-1E2 (serotype 3), and ST-2G7 (serotype 4). All monoclonal antibodies were produced in ascitic fluids. Analyses with groups 1 and 2 were performed at the Gamble Institute, and group 3 was tested at the National Institutes of Health, Bethesda, Md., by using aliquots of the same virus preparations that were analyzed at the Gamble Institute. In addition to these three groups of monoclonal antibodies, an additional monoclonal antibody (3A3) made to serotype 4 strain VA70 (subtype 4B) by Gerna et al. (18) was used for comparative serotyping with serotype 4 isolates.

Serotype determination with monoclonal antibodies. All serotype determinations with monoclonal antibodies were performed by indirect ELISAs. Those conducted at the Gamble Institute included groups 1 and 2 and monoclonal antibody 3A3. These were done by using the monoclonal antibodies to capture viral antigen and serotype-specific polyclonal antibodies to detect viral antigen. Each monoclonal antibody was first tested to determine the dilution needed for maximum sensitivity. Because some monoclonal antibody preparations were found to lose sensitivity at dilutions of >1:500, all were tested at a 1:500 dilution to maintain consistency. Dilutions of monoclonal antibodies were made in high-pH buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.6]), and wells of microtiter plates (MaxiSorb 4-39454; Nunc, Inc., Naperville, Ill.) were coated during overnight incubation (4°C, 50 μl per well). Plates were washed, and tissue culture preparations of viral isolates or laboratory strains of human rotavirus diluted twofold in phosphate-buffered saline (PBS) containing 5% nonfat dry milk (PBS-M) were added (50 μl per well) to duplicate wells of each monoclonal antibody. After incubation (1 h, 37°C), wells were again washed and normal goat serum diluted 1:100 in PBS-M was added (50 μl per well). Following 15 min at room temperature (RT), hyperimmune guinea pig serum against the same serotype as the monoclonal coating antibody (1:200 dilution in PBS-M; 50 μl per well) containing a 1:100 dilution of normal mouse serum was added to one of two duplicate wells. The second well (control) received preimmune guinea pig serum to ensure the specificity of the reaction. After incubation (1 h, RT), wells were washed and biotinylated goat antibody to guinea pig immunoglobulin G (IgG; 1:100 dilution [Vector Laboratories, Burlingame, Calif.] in PBS-M containing a 1:100 dilution of normal mouse serum) was added (50 μl per well) and incubated (30 min, RT). After the plates were washed, avidin preincubated (30 min) with peroxidase-conjugated biotin (1:50 dilution in wash buffer [Vector Laboratories]) was added (50 μl per well), incubated (30 min, RT), and washed from the plates. Substrate (*o*-phenylenediamine) was then added (50 μl per well), the plates were incubated (15 min, RT), and the reaction was stopped by the addition of 1 M H_2SO_4 (75 μl).

The colorimetric reaction was measured by determining the A_{490}, and a specimen was considered positive if the A_{490} value of the well containing hyperimmune serum was at least twice that of the control well and >0.15.

The ELISA conducted at the National Institutes of Health with the group 3 monoclonal antibodies was done in a manner similar to that described by Taniguchi et al. (30). Ascitic fluids, pooled rabbit hyperimmune sera against rotavirus serotypes 1 to 4, and peroxidase-conjugated goat antibody to rabbit IgG were obtained from K. Taniguchi and S. Urasawa. Monoclonal antibody functioned as the capture antibody, and the pooled rabbit hyperimmune serum was the detection antibody. The wells of a microtiter plate were coated with 100 μl of one of the four monoclonal antibodies in group 3 (diluted to 1:10,000 in PBS) overnight at 4°C. After washing with PBS containing 0.05% Tween (PBS-T), 200 μl of blocking buffer (PBS-T–2.5% skim milk) was added, and the plates were incubated at 37°C for 2 h. The plates were washed again, rotavirus antigen or uninfected tissue culture control (50 μl per well) was added, and the plates were incubated for 12 to 18 h at 4°C. After washing with PBS-T, the pooled rotavirus hyperimmune rabbit serum diluted 1:16,000 in PBS-T–2.5% skim milk was added (50 μl per well) and allowed to incubate at 37°C for 1 h. After again washing with PBS-T, goat antibody to rabbit IgG labeled with peroxidase was added (50 μl per well of a 1:10,000 dilution in PBS-T–2.5% skim milk). Following incubation at 37°C for 1.5 h, the wells were washed and substrate [0.1 mg of 2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid) per ml of 0.1 M citrate buffer (pH 5.0), 0.012% hydrogen peroxide] was added (100 μl per well). The A_{405} was measured, and the result was expressed as the average A_{405} in duplicate wells. The background absorbance value in a control well was subtracted from the absorbance value in each of the remaining wells. A specimen was considered to be positive for a particular serotype when its average A_{405} value in duplicate wells with monoclonal antibody corresponding to that serotype was >0.15 and at least twice as high as those with each of the three other serotyping monoclonal antibodies.

Selection of escape mutants. Monoclonal antibodies belonging to group 1 were used to select mutants of laboratory strains that escaped neutralization. Escape mutants of the Wa strain (serotype 1) were selected with either monoclonal antibody 2C9 or monoclonal antibody 5E8, those of the DS-1 strain (serotype 2) were selected with either monoclonal antibody 2F1 or monoclonal antibody 1C10, and escape mutants of the P strain (serotype 3) were selected with monoclonal antibody 159. Serial dilutions (10-fold) of each virus preparation were made in minimum essential medium Dulbecco-modified (D-MEM) and incubated (1 h, 37°C) with an equal volume of monoclonal antibody diluted (1:100) in D-MEM. After neutralization, virus (0.2 ml) was added to confluent monolayers of MA104 cells washed twice to remove serum, and plates were incubated (1 h, 37°C). Unadsorbed virus was then washed from the plates, and medium (D-MEM containing 4 μg of trypsin per ml) with 0.2% agarose was added. Plaques that formed after 3 to 5 days of incubation were picked, and each plate was replaqued either with or without pretreatment with the same monoclonal antibody. Those giving equal numbers of plaques under the two conditions were considered true escape mutants, and preparations of each were made in MA104 cells for further analyses.

Neutralization of escape mutants with different monoclonal antibodies. Escape mutants selected with single monoclonal antibodies were tested to determine whether they escaped neutralization by the other monoclonal antibodies corresponding to the same rotavirus serotype. This was done by using a fluorescence focus reduction neutralization assay (4). Briefly, preparations of each escape mutant containing 5×10^4 to 10×10^4 FFU/ml in D-MEM were incubated (1 h, 37°C) with an equal volume of each monoclonal antibody at a concentration fivefold greater than that required to neutralize 60% of the homologous wild-type strain. After neutralization, virus was diluted 10-fold in D-MEM and added to confluent monolayers of MA104 cells washed twice with D-MEM in 96-well microdilution plates (0.1 ml per well), and the assay was continued as described previously (4). Wells inoculated with virus treated in the same manner but incubated without antibody served as controls. The analysis was performed twice with duplicate wells for each specimen. Average recoveries of FFU were expressed as percentages of control values.

TABLE 1. Properties of the rotavirus isolates included in the study

Serotype	No. of isolates	No. of different electropherotypes
1	44	18
2	23	11
3	21	10
4	55	30

RESULTS

Properties of the culture-adapted rotavirus field isolates selected for study. The serotypes of the 143 culture-adapted rotavirus isolates used in this study were previously determined by neutralization with hyperimmune antisera (37) and included 44, 23, 21, and 55 isolates of serotypes 1 through 4, respectively (Table 1). These were composed of 69 different electropherotypes; and there were 18, 11, 10, and 30 electropherotypes for serotypes 1 through 4, respectively.

Serotype specificity of the monoclonal antibodies used in study. The monoclonal antibodies included in the comparative serotyping study were all found to be serotype specific when tested against laboratory strains representative of the six human rotavirus serotypes. Monoclonal antibodies belonging to groups 1 and 2 were tested at the Gamble Institute (Table 2). The specificities of the group 3 monoclonal antibodies, i.e., KU6BG, S2-2G10, YO-1E2, and ST-2G7, were determined at the National Institutes of Health, as stated above, and the results are described elsewhere (18a).

Comparative serotype analysis of field isolates with monoclonal antibodies. When tested with the 143 field isolates of rotavirus, all monoclonal antibodies reacted only with the isolates that corresponded to the serotype predicted by neutralization with polyclonal antisera (Table 3). Eight isolates found to be neutralized by polyclonal antisera to more than one serotype reacted only with the monoclonal antibody that corresponded to the dominant serotype. Although the specificities of these monoclonal antibodies in serotype determination were all 100%, their sensitivities were very different (Table 3). For example, monoclonal antibody 5E8 reacted with 41 of 44 (93%) of the serotype 1 isolates, while monoclonal antibody 2C9 reacted with only 1 of 44 (2%) of the serotype 1 isolates. Further analyses were conducted to determine the causes for these observed differences.

The inability of monoclonal antibody 5E8 to react with three isolates appeared to be due to insufficient intact

TABLE 2. Reactivities of group 1 and 2 serotyping monoclonal antibodies with representatives of the six serotypes of human rotavirus determined by ELISA

Group and monoclonal antibody	Serotype[a]	Absorbance value[b]			
		Wa (1)	DS-1 (2)	P (3)	ST-3 (4)
Group 1					
5E8	1	1.651			
2C9	1	0.458			
2F1	2		0.542		
1C10	2		0.916		
159	3			1.384	
Group 2					
RV4:2	1	0.217			
RV5:3	2		0.824		
RV3:1	3			1.077	
ST-3:1	4				0.952

[a] Serotype specificity of the monoclonal antibody.
[b] Absorbance values are the differences between A_{490} in wells containing hyperimmune and preimmune (control) guinea pig serum (see text). Values were given only if the A_{490} in the well with hyperimmune serum was >0.15 and at least two times the A_{490} of the control well. By these criteria, there were no values for monoclonal antibodies with strain 69M (serotype 8) or WI61 (serotype 9). Serotypes of rotavirus strains are given in parentheses.

particles in these viral preparations. The titers of the serotype 1 preparations ranged from 3×10^3 to 1×10^7 FFU/ml, and the three isolates not detected by 5E8 were among the four with the lowest titers (Table 4). Furthermore, additional isolates that belonged to the same electropherotypes all reacted with 5E8. The inability of monoclonal antibody 2C9 to react, on the other hand, appeared to be due to the lack of proper epitopes on nonreactive isolates. The one specimen with which 2C9 reacted had an intermediate titer (3×10^6 FFU/ml), and other isolates with higher titers gave stronger reactions with 5E8.

Six serotype 1 isolates did not react with monoclonal antibody RV4:2; and five of these six, i.e., isolates 34, 49, 52, 95, and 396, were those with the lowest titers (Table 4). When the same preparations of these five isolates were tested under the conditions described by Coulson et al. (9), only the isolate with the lowest titer (isolate 34) did not react with RV4:2. Thus, the inability to react with these five isolates was apparently due to insufficient intact particles. The inability of RV4:2 to react with the sixth isolate (isolate 26), however, appeared to be due to a lack of the proper epitope. This virus preparation contained 9×10^6 FFU/ml and readily reacted with monoclonal antibody 5E8 but did not react with monoclonal antibody RV4:2 under the assay conditions used in this study or those of Coulson et al. (9). Another isolate with the same electropherotype as that of isolate 26 but a titer of only 2×10^5 FFU/ml did react with RV4:2, thus demonstrating epitope variation within the same electropherotype.

The group 3 monoclonal antibody to serotype 1 strains, KU6BG, also did not react with three of the four serotype 1 isolates with the lowest titers nor with isolate 26 (Table 4). In addition, this monoclonal antibody failed to react with isolate 23 but did react with two other isolates with the same electropherotype as that of isolate 23. Because all three had titers of approximately 2×10^6 FFU/ml, an inability to react with isolate 23 was also apparently due to the absence of the KU6BG epitope on this isolate. Thus, none of the four monoclonal antibodies to serotype 1 strains examined in this study appeared to be directed against identical epitopes.

Serotype 2 isolates had titers that ranged from 4×10^3 to 2×10^7 FFU/ml, and the group 1 monoclonal antibody 1C10 reacted with all but the isolate (isolate 57) with the lowest titer (Table 4). The other group 1 monoclonal antibody, 2F1, reacted with only 5 of 23 (22%) of serotype 2 isolates, and these were the five that gave the strongest reaction with monoclonal antibody 1C10. In addition, these five isolates were among the eight isolates with the highest concentrations of infectious particles, and other isolates with the same electropherotypes but lower titers did not react with 2F1. Thus, the lack of sensitivity of 2F1 appeared to be due at

TABLE 3. Reactivities of serotyping monoclonal antibodies with 143 culture-adapted human rotavirus isolates

Serotype and monoclonal antibody	No. of isolates with reactivity[a]				Sensitivity (%)[b]
	Serotype 1 (44)	Serotype 2 (23)	Serotype 3 (21)	Serotype 4 (55)	
Serotype 1					
5E8	41	0	0	0	93
2C9	1	0	0	0	2
RV4:2	38	0	0	0	86
KU6BG	39	0	0	0	89
Serotype 2					
2F1	0	5	0	0	22
1C10	0	22	0	0	96
RV5:3	0	19	0	0	83
S2-2G10	0	19	0	0	83
Serotype 3					
159	0	0	21	0	100
RV3:1	0	0	21	0	100
YO-1E2	0	0	0	0	0
Serotype 4					
ST-3:1	0	0	0	55	100
ST-2G7	0	0	0	54	98

[a] Numbers of isolates belonging to each serotype, as determined by neutralization with polyclonal antibodies, are given in parentheses.
[b] Sensitivity is (number of actual positives/number of true positives) × 100.

TABLE 4. Titers of rotavirus isolates not reactive with monoclonal antibodies of the corresponding serotypes

Serotype and monoclonal antibody	Nonreactive isolates of corresponding serotype	Titer of nonreactive isolates (FFU/ml)
Serotype 1		
5E8	34, 49, 52	3×10^3–3×10^4
2C9	All but 63	3×10^3–1×10^7
RV4:2	34, 49, 52, 95, 396	3×10^3–7×10^4
RV4:2	26	9×10^6
KU6BG	34, 52, 95	3×10^3–3×10^4
KU6BG	23, 26	2×10^6–9×10^6
Serotype 2		
2F1	All but 3, 10, 11, 328, 456	4×10^3–5×10^6
1C10	57	4×10^3
RV5:3	57	4×10^3
RV5:3	288, 290, 317	7×10^5–1×10^6
S2-2G10	57	4×10^3
S2-2G10	288, 290, 317	7×10^5–1×10^6
Serotype 3		
159	None	
RV3:1	None	
YO-1E2	All	1×10^5–1×10^7
Serotype 4		
ST-3:1	None	
ST-2G7	92	9×10^4

least partially to the binding affinity of this monoclonal antibody. The group 2 and 3 monoclonal antibodies to serotype 2 strains, RV5:3 and S2-2G10, also did not react with isolate 57. In addition, neither reacted with three other serotype 2 isolates (isolates 288, 290, and 317), all of which had titers of 7×10^5 to 1×10^6 FFU/ml and belonged to the same electropherotype. Because these were the only isolates with this electropherotype, it appeared that they belonged to a rotavirus strain that lacked the epitope(s) recognized by RV5:3 and S2-2G10.

The 21 serotype 3 isolates included in this study had titers of between 10^5 and 10^7 FFU/ml, and all reacted with the group 1 and 2 monoclonal antibodies to serotype 3 strains, i.e., 159 and RV3:1 (Table 4). In contrast, the group 3 monoclonal antibody to serotype 3, YO-1E2, did not react with any of these isolates. Thus, the epitope recognized by YO-1E2 was apparently not present in any of the 21 rotavirus isolates tested in this study.

Titers for the serotype 4 preparations ranged from 2×10^4 to 3×10^7 FFU/ml, and all isolates gave a strong reaction with the group 2 monoclonal antibody to this serotype, ST-3:1 (Table 4). The group 3 monoclonal antibody ST-2G7 failed to react with only one of these isolates (isolate 92). This isolate belonged to the same electropherotype as that of the two other serotype 4 strains, but the titer of isolate 92, i.e., 9×10^4 FFU/ml, was ≥20-fold less than those of the other two isolates. This suggested that the isolate 92 rotavirus preparation has too few infectious particles to react with ST-2G7.

Subtypes of serotype 4 isolates. It has been reported that serotype 4 human rotaviruses can be divided into two subtypes represented by the asymptomatic neonatal strain ST-3 (subtype 4A) and the symptomatic strain VA70 (subtype 4B) (18). Serotype 4 isolates have been shown to react almost exclusively with monoclonal antibodies generated against one but not the other of these prototype strains. Although the serotype 4 strains examined in this study were neutralized by hyperimmune antiserum to ST-3, the neutralization titers of polyclonal anti-ST-3 antibody to these isolates are 28- to 200-fold less than that to the homologous virus (37). On the other hand, the homologous virus/heterologous virus ratios of hyperimmune anti-VA70 antibody to these isolates ranged between 2 and 40 (median; 5). Although these isolates appeared to be much more closely related to VA70 than to ST-3, they readily reacted with monoclonal antibodies to ST-3, i.e., ST-3:1 and ST-2G7. It was therefore of interest to determine whether a subtype-specific monoclonal antibody to VA70, 3A3, would also react with these isolates.

Monoclonal antibody 3A3 was found to react with VA70 but not with ST-3 (data not shown). However, 3A3 did not react with any of the 55 serotype 4 isolates included in this study, a result confirmed by Gerna et al. (18) by a subtyping ELISA. Therefore, based on their reactivities with monoclonal antibodies ST-3:1 and 3A3, these serotype 4 isolates all belong to subtype 4A.

Use of escape mutants to predict similarities and differences in epitope specificities of monoclonal antibodies. Based on the reactivities of the different monoclonal antibodies with the rotavirus isolates examined in this study, it appeared that certain monoclonal antibodies to the same serotype recognize different epitopes. For example, none of the four monoclonal antibodies to serotype 1 rotaviruses appeared to recognize identical epitopes. To test this hypothesis, mutants of the serotype 1 human rotavirus strain Wa that escaped neutralization by either monoclonal antibody 2C9 or monoclonal antibody 5E8 were selected. These, in turn, were tested to determine whether they also escaped neutralization by the other group 1 and 2 monoclonal antibodies to serotype 1 strains; group 3 monoclonal antibodies were not available for this analysis. If an escape mutant with one monoclonal antibody also escaped neutralization by a second monoclonal antibody, it indicated that the epitopes recognized by these antibodies are overlapping, if not identical. If an escape mutant selected with one monoclonal antibody was neutralized by a second monoclonal antibody, however, it clearly shows that the monoclonal antibodies recognize different, although possibly overlapping, epitopes.

Five Wa mutants that escaped neutralization by monoclonal antibody 5E8 all escaped neutralization by 2C9 and RV4:2 (Table 5). This suggested that the epitopes recognized by these three monoclonal antibodies are at least overlapping, if not identical. However, when eight Wa escape mutants to 2C9 were tested with 5E8 and RV4:2, only one of the eight (2C9-7) partially or completely escaped neutralization by these two monoclonal antibodies. This indicated that all three monoclonal antibodies recognize somewhat different epitopes, as predicted from the ELISA results.

Four escape mutants of the serotype 2 DS-1 strain selected by monoclonal antibody 1C10 also escaped neutralization by 2F1 and RV5:3 (Table 5), which suggested that all three monoclonal antibodies recognize overlapping, if not identical, epitopes. However, two of the four escape mutants selected by 2F1 (2F1-3 and 2F1-5) escaped neutralization by RV5:3 but not 1C10, one (2F1-4) escaped neutralization by both RV5:3 and 1C10, and one (2F1-1) did not escape neutralization by either. Thus, all group 1 and 2 monoclonal antibodies to serotype 2 apparently recognize different, although probably overlapping, epitopes.

All 21 serotype 3 isolates included in this study reacted with group 1 and 2 monoclonal antibodies to serotype 3 strains (Table 3). This suggested that monoclonal antibodies

TABLE 5. Abilities of rotavirus escape mutants selected by specific monoclonal antibodies to escape neutralization by other monoclonal antibodies to the same serotype

Parental rotavirus strain	Selecting monoclonal antibody	Mutant no.	% Recoverable infectious rotaviruses (FFU) for the following serotypes and indicated monoclonal antibodies[a]							
			Serotype 1			Serotype 2			Serotype 3	
			5E8	2C9	RV4:2	1C10	2F1	RV5:3	159	RV3:1
Wa		Wa	5	3	9					
	5E8	1, 2, 3, 4, 5	100	100	100					
	2C9	1, 2, 3, 4, 5, 6, 8	4–10	100	4–9					
	2C9	7	39	100	100					
DS-1		DS-1				20	12	2		
	1C10	1, 2, 5, 6				100	100	100		
	2F1	3, 5				18–23	100	100		
	2F1	4				100	100	100		
	2F1	1				20	100	1		
P		P							0	3
	159	1, 2							100	100

[a] Results are expressed as the percentage of recoverable FFU relative to that of untreated controls after neutralization of parental strains or escape mutants with five times the concentration of antibody required to reduce the titer of the parental strain by 60%. Values are averages of two experiments. The number of FFU per well in the absence of antibody (controls) varied for each virus but ranged from 170 to 840.

159 and RV3:1 recognize the same epitope. Consistent with this suggestion, it was found that the two escape mutants of the serotype 3 P strain selected with monoclonal antibody 159 also escaped neutralization by RV3:1 (Table 5).

DISCUSSION

Serotyping of human rotavirus isolates by ELISA with serotype-specific monoclonal antibodies is a rapid method of viral identification that has been used in numerous studies with varying degrees of success. It has been suggested that the absence of sufficient intact viral particles in stool specimens of infected subjects is primarily responsible for limiting the usefulness of this procedure (9, 17, 25, 31, 32). It has also been found that epitopes reactive with some serotyping monoclonal antibodies are not present on certain rotavirus isolates of the corresponding serotype (6, 8, 25, 31). The existence of such monotypes (8) suggests the possibility that additional monoclonal antibodies may be needed for consistent serotype determination by this method. Other factors, such as inhibition of the ELISA by components in the stool specimen, could also limit the usefulness of the assay.

Three groups of monoclonal antibodies from different sources were used in this comparative serotyping study. These three were chosen primarily because each has been distributed worldwide and has been used by numerous laboratories in past studies. When tested by serotyping ELISAs with 143 culture-adapted rotavirus isolates belonging to 69 different electropherotypes, all monoclonal antibodies of these three groups gave results that were 100% consistent with those found previously by neutralization with polyclonal antibody preparations. That is, no false-positive results were observed.

Eight isolates included in this study were found to be neutralized by polyclonal antibody to more than one serotype, but in every case a dominant serotype was readily distinguished. Serotyping monoclonal antibodies reacted only with the dominant electropherotype. These eight isolates may be serotypic mosaics that derived their VP7 protein gene from the dominant serotype and their gene for the VP4 protein, the other rotavirus neutralization protein, from a different serotype of rotavirus, presumably through gene reassortment. One of these eight isolates (isolate 248) was examined in greater detail. It was found to react only with monoclonal antibodies to serotype 4 strains but was neutralized by polyclonal antibodies to serotypes 2 and 4 (36). By RNA-RNA hybridization analysis, it was shown to be an intergenogroup reassortant; its VP7 gene was derived from a rotavirus belonging to the ST-3 (Wa) genogroup, and its VP4 gene was from a virus belonging to the DS-1 genogroup.

Large variations in the abilities of different serotyping monoclonal antibodies to react with rotaviruses of the corresponding serotypes were noted in this study. In some cases, it was found that lack of reactivity was the result of low titers of infectious particles in the virus preparations. Serotype 1 monoclonal antibodies 5E8, RV4:2, and KU6BG did not react with several isolates, apparently for this reason. Similarly, it appeared that all four serotype 2 monoclonal antibodies tested and the serotype 4 monoclonal antibody ST-2G7 also did not react with certain isolates for the same reason.

Lack of reactivity in other instances appeared to be due to epitope variation within viral isolates. Monoclonal antibody RV4:2 failed to react with one serotype 1 isolate (isolate 26) that had a high titer, and monoclonal antibody KU6BG did not react with this isolate nor with one additional serotype 1 isolate (isolate 23), which also had a high titer. Furthermore, monoclonal antibody 2C9 reacted strongly with the prototype Wa strain but reacted with only 1 of the 44 serotype 1 strains in this study. A similar finding was reported by Padilla-Noriega et al. (25) with rotavirus specimens obtained in Mexico. These results indicated that the epitope recognized by 2C9 was present on few serotype 1 isolates (i.e., monotypes) and that epitopes recognized by RV4:2 and KU6BG were also not present on all serotype 1 human rotaviruses included in this study. In fact, all four monoclonal antibodies specific for serotype 1 appeared to react with different epitopes. This conclusion was supported by results found with mutants of the Wa strain that escaped neutralization by 2C9. Seven of eight 2C9 escape mutants did not escape neutralization by either 5E8 or RV4:2, but mutant

2C9-7 totally escaped neutralization by RV4:2 and partially escaped neutralization by 5E8. Because all five Wa escape mutants selected by 5E8 also escaped neutralization by 2C9 and RV4:2, the epitopes recognized by these monoclonal antibodies probably overlap. RNA sequencing has been conducted on the VP7 protein gene of a 2C9 escape mutant, and a single amino acid change was detected at position 94 (23). This site appeared to be part of a major neutralization epitope in a number of rotavirus strains (23).

Epitope variation also appeared to be responsible for lack of reactivity between monoclonal antibodies RV5:3 and S2-2G10 with three serotype 2 isolates, all with the same electropherotype. Because monoclonal antibody 1C10 reacted strongly with all three isolates, this monoclonal antibody apparently recognized a somewhat different epitope. Cross-neutralization studies with escape mutants of the DS-1 serotype 2 strains selected with 2F1 indicated that 2F1, 1C10, and RV5:3 were not directed against identical epitopes. Sequence analysis of the VP7 protein gene of these escape mutants is needed to determine the amino acid changes responsible for these observed differences.

Lack of reactivity because of epitope variation was most evident with the serotype 3 monoclonal antibody YO-1E2. This monoclonal antibody failed to react with any of the 21 serotype 3 isolates examined, which represented 10 different electropherotypes. Thus, the epitope recognized by this antibody was apparently present in few, if any, isolates collected in Bangladesh during the period of the study. Using the group 3 monoclonal antibodies, others (26) reported finding no serotype 3 isolates in the same geographic region (Thailand) in rotavirus isolates collected during either 1983 to 1984 or 1987 to 1988, even though serotype 1, 2, and 4 isolates were readily detected. Likewise, only serotype 1, 2, and 4 human rotaviruses were detected with these same serotyping monoclonal antibodies in specimens collected in Israel during 1986 to 1987 (10). Furthermore, Padilla-Noriega et al. (25) found that monoclonal antibody YO-1E2 reacted with only 5 of 13 serotype 3 rotavirus specimens collected in Mexico, all of which reacted with monoclonal antibody 159. Thus, even though group 3 monoclonal antibodies have been used in many serotyping studies, the serotype 3 antibody of this group may react with only a small fraction of serotype 3 strains. General use of YO-1E2 alone in such studies should be reevaluated.

The other serotype 3 monoclonal antibodies used in this study, 159 and RV3:1, reacted with all 21 serotype 3 isolates. Escape mutants of the P strain selected with monoclonal antibody 159 also escaped neutralization by RV3:1. This suggested that the two are directed against overlapping, if not identical, epitopes. Escape mutants of serotype 3 simian rotaviruses selected with 159 and RV3:1 have been mapped in VP7 amino acid positions 94 and 211, respectively (11, 22). Because these distal sites appear to fold together as a single conformational unit in native VP7 (11, 23, 29), the epitopes recognized by 159 and RV3:1 may still be identical.

The results of this study also indicated that the serotype 4 isolates all belong to subtype 4A because they reacted with monoclonal antibodies to ST-3, the prototype for this rotavirus subtype, and none reacted with monoclonal antibody 3A3 made to the subtype 4B strain VA70. Interestingly, neutralization of these isolates by polyclonal antibody to ST-3 or VA70 indicated that they are much more closely related to the latter (37). This suggested that the epitopes recognized by the subtype 4A monoclonal antibodies ST-3:1 and ST-2G7 are not immunodominant during production of hyperimmune antiserum to ST-3. These results also suggested that a subtype 4A epitope can be present on rotavirus strains that appear to be more closely related to subtype 4B strains, as determined with hyperimmune antisera.

From the results obtained in this study, it appears that certain monoclonal antibodies can be used to reliably determine the serotypes of most, if not all, human rotaviruses belonging to that serotype. For example, monoclonal antibody 5E8 appeared to react with all serotype 1 strains with sufficient infectious viral particles. However, strain Wa mutants could be selected that escaped neutralization by this monoclonal antibody, which suggests that not all serotype 1 strains necessarily contain the 5E8 epitope. Therefore, even though the results of this study indicate that single monoclonal antibodies to each serotype may be sufficient to determine the serotypes of all or nearly all human rotavirus isolates, geographic and seasonal variations in rotavirus strains could still yield variants that are not typeable with certain monoclonal antibodies. In this case, additional monoclonal antibodies that recognize different epitopes may be needed for efficient serotype determination.

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