Identification of Two Subtypes of Serotype 4 Human Rotavirus by Using VP7-Specific Neutralizing Monoclonal Antibodies

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Two distinct subtypes of human rotavirus serotype 4 were identified by using neutralizing monoclonal antibodies directed to the major outer capsid glycoprotein, VP7, of strains ST3 (subtype 4A) and VA70 (subtype 4B). Specimens containing serotype 4 rotavirus, obtained from different countries, were examined for subtyping by using solid-phase immune electron microscopy, enzyme-linked immunosorbent assay, and, for cell culture-adapted strains, neutralization assay. All 59 human rotavirus strains identified as serotype 4 by using animal antisera were classified into either subtype by monoclonal antibodies. This suggests that the antigenic difference between the two subtypes is a consequence of critical variations within the immunodominant serotype 4-specific neutralization site of rotavirus VP7. Subtype 4A (ST3-like) strains were predominant and were detected in stools from patients with gastroenteritis, as well as from healthy infants and young children.

Group A human rotavirus (HRV) strains, the principal cause of acute nonbacterial gastroenteritis in infants and young children, are classified into four distinct serotypes on the basis of their specific reactivity with animal hyperimmune sera in neutralization (NT) assays (12). In the last few years, two presumptive new HRV serotypes have been identified in cross-NT assays (1, 3, 15). The rotavirus classification proposed by Hoshino et al. (12), which has been widely accepted, included HRV serotypes 1 to 4, porcine rotavirus (OSU strain) as serotype 5, bovine rotavirus as serotype 6, and avian rotavirus as serotype 7. The two new distinct HRV serotypes, once confirmed as such, will possibly be classified as serotypes 8 and 9. The major antigen responsible for rotavirus serotype specificity was believed to be the outer capsid glycoprotein VP7, the eighth or ninth gene product (10, 13, 16, 18). However, recently the outer capsid protein VP3, coded for by the fourth rotavirus gene, was shown to be capable of eliciting neutralizing antibody at a level as high as that elicited by VP7 (1, 17). Thus, in the future a better definition of rotavirus serotypes should include designation of both VP3 and VP7 specificities.

In 1985, using cross-absorbed animal polyclonal immune sera and the solid-phase immune electron microscopy (SPIEM) technique, we identified two distinct subtypes within serotype 4 HRV strains (8). The two reference strains, ST3 (subtype 4A) and VA70 (subtype 4B), both appeared to be serotype 4 by NT assay but were clearly differentiated by SPIEM, and all serotype 4 HRV strains were classified into either subtype. However, after absorption with all heterotypes, ST3 antiserum was reactive with both subtypes, whereas VA70 antiserum was highly subtype specific. In the present study, by using neutralizing mouse monoclonal antibodies (MAbs) specific to VP7 of either ST3 or VA70, the two subtypes of serotype 4 HRV have definitely been identified by enzyme-linked immunosorbent assay (ELISA), SPIEM, and, for cell culture-adapted strains, NT, thus suggesting that critical variations between the two

subtypes occur within the immunodominant serotype 4-specific NT site of VP7.

MATERIALS AND METHODS

Rotavirus strains. Cell culture-adapted rotavirus strains DS-1, Wa, and ST3 were kindly provided by R. G. Wyatt, National Institute of Allergy and Infectious Diseases, Bethesda, Md. Strain YO was supplied by S. Urasawa, University of Sapporo, Sapporo, Japan. Strains VA70, VA75, VA79, PV5249, and PV5257 were previously adapted to growth on cell cultures in our laboratory (7). All strains were propagated on MA104 cell cultures in the presence of trypsin (7).

Antisera. Immune sera against reference HRV serotypes DS-1, Wa, YO, ST3, and VA70 and several other cell culture-adapted HRV strains were prepared in our laboratory by immunizing rabbits and guinea pigs, free of rotavirus antibody, with partially purified virus (7). For use in the SPIEM assay, sera from immunized animals were made type specific by sequential absorption steps (8). Briefly, each antiserum was first absorbed with single-shelled rotavirus particles of subgroups I and II and then with a mixture of double-shelled virus particles of the three heterotypes. After a final centrifugation at 140,000 × g for 90 min to pellet the virus, absorbed sera were suitable for typing, i.e., free of group, subgroup, and heterotypic reactivity.

Murine MAbs. Rotavirus type-specific neutralizing MAbs RV-4:2 (anti-serotype 1), RV-5:3 (anti-serotype 2), RV-3:1 (anti-serotype 3), and ST-3:1 (anti-serotype 4) were provided by B. S. Coulson, University of Melbourne, Parkville, Victoria, Australia. Their properties have previously been extensively reported (4, 5). MAbs to HRV 4B were produced in our laboratory essentially as described for SA11-neutralizing MAbs (8a) but by using BALB/c mice immunized with partially purified VA70 and VA70-like strains. Hybridomas showing NT activity were subcloned twice by limiting dilution and then grown as ascites tumors in Pristane-primed syngeneic mice. Neutralizing MAbs were then titrated against a panel of serotype 4A and 4B HRVs previously

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adapted to growth on cell cultures. In parallel, the immuno-logical reactivity of MAbs with reference HRV strains of serotypes 1, 2, and 3 was also examined by NT and ELISA. The immunoglobulin isotype of VA70 MAbs was determined by ELISA (Mouse-Typer Sub-Isotyping Kit; Bio-Rad Laboratories, Richmond, Calif.).

Western blot (immunoblot) analysis. Sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis in reducing conditions was performed as reported by Laemmli (14). For analysis of unreduced proteins, samples were solubilized in Laemmli sample buffer without 2-mercaptoethanol. For Western blotting, proteins were transferred from gels onto 0.2-μm-pore-size nitrocellulose paper essentially as described by Towbin et al. (19). Immunological detection of blotted proteins was performed by the avidin-biotin peroxidase complex procedure (Vectastain ABC Kit; Vector Laboratories, Burlingame, Calif.).

NT assay. The NT assay was performed with MA104 cell cultures grown in microdilution plates as previously described (7). Briefly, equal volumes of a trypsin-treated virus suspension (approximately 200 focus-forming units per 0.1 ml) and serial dilutions of ascitic fluids were incubated for 30 min at 37°C and then inoculated onto MA104 cell monolayers. After incubation at 37°C for 18 h, cells were fixed and stained by the immunoperoxidase technique for the detection of non-neutralized virus. The NT titer was expressed as the reciprocal of the highest dilution giving at least 50% reduction in the number of infected cells as compared with the virus control wells.

ELISA. Rotavirus suspensions of known serotypes as well as negative control suspensions were prepared in phosphate-buffered saline, pH 7.2, containing 0.1% (vol/vol) Tween 20–10% fetal calf serum. Specimens were incubated for either 2 h at 37°C or overnight at 4°C in microdilution plate wells previously coated with homotypic polyclonal antibody (diluted 1:10,000) and with preimmune immunoglobulin. After washing, optimal dilutions of MAbs, as determined by checkerboard titration against homologous strains, were added and the mixture was incubated for 2 h at 37°C. Biotinylated antibody to mouse immunoglobulin was then added. After a 90-min incubation at 37°C, the bound biotinylated antibody was detected by adding avidin-biotin peroxidase complex. After incubation for 30 min at 37°C, o-phenylenediamine was added as a chromogenic substrate and the mixture was incubated for 30 min at room temperature. The A_{492} was determined in a microdilution plate reader (Titertek, Multiskan; Flow Laboratories, Irvine, Scotland). Negative control specimens were consistently unreactive (A_{492}, <0.05). The reactivity of specimens in wells coated with preimmune immunoglobulin was also negligible (A_{492}, <0.05). HRV strains were considered typed when the differential A_{492} between immune- and preimmune-immunoglobulin-coated wells was >0.10.

SPIEM. The SPIEM technique was performed essentially as reported previously (8). Type-specific MAbs to HRV serotypes 1, 2, 3, 4A, and 4B were titrated by SPIEM to determine their optimal working dilutions. These were then used for typing reference HRV strains, previously typed by NT and SPIEM that used absorbed polyclonal antibody (8). Briefly, carbon-Formvar-coated 400-mesh grids were floated on 25 μl of protein A solution (25 μg/ml) for 10 min and then on each of the five type- and subtype-specific MAbs for 20 min. Previous incubation of protein A-coated grids with rabbit anti-mouse immunoglobulin (30 min at room temperature) was required for binding of 4B-specific MAb (immunoglobulin G1 isotype). Finally, the grids were floated overnight on virus

suspensions. After negative staining, 20 grid squares were examined for each grid with a Philips EM201 electron microscope at ×45,000, and the average number of virus particles per grid square was determined. Typing ratios of rotavirus strains were calculated as described previously (8).

Serotype 4 HRV strains. A group of 59 serotype 4 HRV strains previously serotyped by NT or SPIEM or both was examined for subtyping. The specimens were from several European countries, North and South America, and Australia. Viral RNA was extracted from some samples and analyzed by polyacrylamide gel electrophoresis for determination of electropherotype (6).

RESULTS

Subtyping of HRV serotype 4 by using animal antisera. When ST3 and VA70 hyperimmune sera were tested in cross-NT assays, HRV strains ST3 and VA70 appeared to be antigenically different, even though such a difference was not sufficient to define two distinct serotypes, according to established criteria (12). In SPIEM, after cross-absorption of hyperimmune sera to make them type specific (8), ST3 antiserum strongly reacted with both ST3 and VA70 strains, whereas VA70 antiserum did not appreciably react with ST3 virus. Thus, ST3 could be typed only by homologous antiserum in SPIEM, whereas VA70 was typed by both antisera. However, ST3 immune serum could correctly type many, but not all, of the VA70-like HRV strains available. As a result, both subtype 4A and subtype 4B antisera had to be used in the SPIEM typing assay to provide adequate sensitivity.

Reactivity of subtype-specific MAbs. The results of cross-NT tests for characterization of MAbs are shown in Table 1. ST3 MAb cross-reacted with VA70 HRV at a dilution of 1:1,000 but neutralized homologous virus at a 300-fold-higher titer. On the other hand, VA70 MAb was not cross-reactive with the ST3 strain.

The two serotype-specific MAbs were examined by ELISA, in which fourfold dilutions of each MAb were reacted with different amounts of homologous and heterologous virus bound to a solid phase previously coated with homologous polyclonal antibodies. The results (Fig. 1) resembled those obtained by NT. In other words, ST3 MAb cross-reacted with VA70 virus at low dilutions (1:2,500 to 1:10,000), but its cross-reactivity disappeared at higher dilutions. VA70 MAb, on the other hand, appeared to be highly subtype specific even at relatively low dilutions.

The cross-reactivity of ST3 MAb was confirmed by Western blot analysis (Fig. 2). Although neither MAb reacted with rotavirus proteins separated under reducing conditions, both MAbs reacted with unreduced VP7 of homologous virus. However, ST3 MAb reacted, weakly, with unreduced

TABLE 1. Cross-NT tests on HRV reference strains by using MAbs

MAb to HRV strain (serotype)	NT antibody titer to HRV strain (serotype)[a]:				
	DS-1 (2)	Wa (1)	YO (3)	ST3 (4A)	VA70 (4B)
DS-1 (2)	**30,000**	<1,000	<1,000	<1,000	<1,000
Wa (1)	<1,000	**10,000**	<1,000	<1,000	<1,000
YO (3)	<1,000	<1,000	**100,000**	<1,000	<1,000
ST3 (4A)	<1,000	<1,000	<1,000	**300,000**	1,000
VA70 (4B)	<100	<100	<100	<100	**30,000**

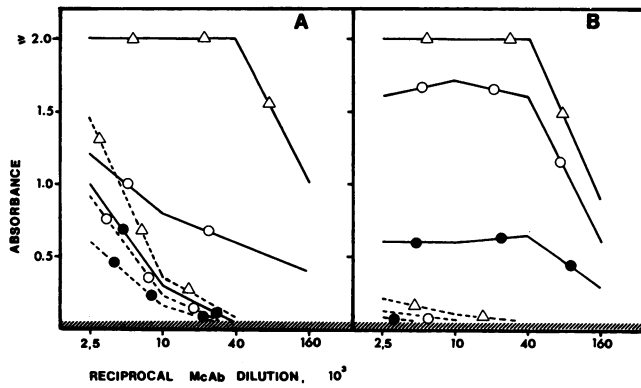[a] Titers are expressed as reciprocals. Homologous titers are given in boldface.

FIG. 1. ELISA reactivity of MAbs to serotype 4 HRV strains ST3 (subtype 4A) and VA70 (subtype 4B). Fourfold dilutions of each MAb were tested against high (10 μg per well) (△), intermediate (1 μg per well) (O), and low (0.1 μg per well) (●) amounts of virus. (A) ST3 MAb; (B) VA70 MAb. Other symbols: ———, homologous reactivity; ---, heterologous reactivity.
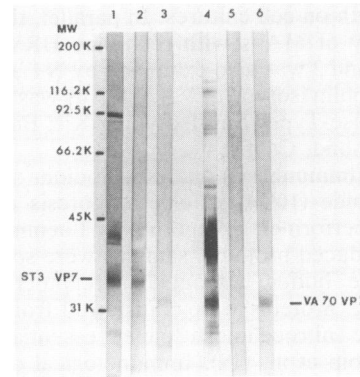

FIG. 2. Western blot reactivity of MAbs with unreduced proteins of serotype 4 HRV strains ST3 (subtype 4A) and VA70 (subtype 4B). ST3 proteins were incubated with homologous antiserum (lane 1), ST3 MAb (lane 2), and VA70 MAb (lane 5); VA70 proteins were reacted with ST3 MAb (lane 3), homologous antiserum (lane 4), and VA70 MAb (lane 6). Molecular weights (10^3) of marker proteins are indicated on the left.

VP7 of strain VA70, whereas VA70 MAb appeared to be subtype specific. In addition, unreduced VP7 of ST3 HRV consistently showed a slower electrophoretic migration than that of VA70 virus; the apparent molecular weights were 34,000 and 32,000 for ST3 and VA70, respectively.

Subtyping of serotype 4 strains by using MAbs. When used in the SPIEM system for typing HRVs, ST3 MAb was able to type as serotype 4 both ST3 and VA70 strains, whereas VA70 MAb appeared to be more subtype specific. However, ST3 MAb identified VA70 virus as serotype 4 with a typing ratio close to the cutoff value of 4.0 (8), and in about one-half of repeated tests the cutoff typing ratio was not reached. Consequently, when the two MAbs were reacted in SPIEM with several serotype 4 HRV strains (previously typed by NT and SPIEM by using animal antisera), it was found that most VA70-like (subtype 4B) strains were not recognized by ST3 MAb, whereas they were all identified by VA70 MAb. On the other hand, ST3-like (subtype 4A) strains were recognized only by ST3 MAb. The subtype was better defined by calculating a subtyping ratio, obtained by dividing the number of double-shelled rotavirus particles trapped by ST3 MAb by the number of particles trapped by VA70 MAb (Table 2). Of the 59 serotype 4 strains examined, 46 (78.0%) were subtype 4A and 13 (22.0%) were subtype 4B (Table 3). The subtyping ratios were >3.00 for subtype 4A strains and between 0.05 and 0.30 for subtype 4B HRVs.

An ELISA was developed for subtyping serotype 4 HRV strains, using MAbs. It was found that serotype 4 reference strains could be simultaneously typed and subtyped by using optimal dilutions of both ST3 and VA70 MAbs and MAbs specific for HRV serotypes 1 to 3 (Table 4). Actually, to type all HRV 4 strains, typing and subtyping had to be done at the same time, using both MAbs. Otherwise, the HRV specimens poor in double-shelled rotavirus particles could often not be identified as serotype 4, and thus they would have been considered untypable unless tested with both subtype-specific MAbs. Results of HRV subtyping by ELISA were in complete agreement with those obtained by SPIEM (Tables 2 and 3). ELISA subtyping ratios, calculated for each strain by dividing the absorbance in wells reacted with ST3 MAb by that in the wells reacted with VA70 MAb, were >3.00 for subtype 4A HRVs and between 0.03 and 0.15 for subtype 4B strains.

A few cell culture-adapted HRV strains of serotype 4 were available. By using ST3 and VA70 MAbs, they could be subtyped also by NT. Results of subtyping by NT assay were in agreement with those obtained by SPIEM and ELISA (data not shown).

Prevalence of serotype 4 subtypes. Specimens containing serotype 4 HRVs from several countries were available. Subtype 4A strains were found to circulate in all areas of the three continents from which specimens were collected.

TABLE 2. Subtyping of 10 serotype 4 HRV strains by SPIEM and ELISA, using MAbs

Serotype 4 HRV strain	No. (10^2) of viral particles per grid square by SPIEM, using MAb to serotype 4 HRV strain (subtype):		SPIEM ratio	ELISA reactivity (A_{492}) by MAb to serotype 4 HRV strain (subtype):		ELISA ratio	Subtype
	ST3 (4A)	VA70 (4B)		ST3 (4A)	VA70 (4B)		
ST3	20.0	0.4	50.0	1.34	0.04	33.50	4A
VA70	8.0	52.0	0.15	0.10	2.00	0.05	4B
Hochi	46.0	6.0	7.66	4.50	1.23	3.65	4A
VA75	1.6	20.0	0.08	0.33	4.50	0.07	4B
VA79	5.0	17.6	0.28	0.21	4.00	0.05	4B
VA101	8.0	1.2	6.66	6.00	1.91	3.14	4A
VA106	11.6	50.0	0.23	0.55	6.00	0.09	4B
ER44	12.8	2.4	5.33	4.50	1.16	3.87	4A
PV5249	26.0	8.0	3.25	1.19	0.10	11.90	4A
PV5257	8.0	32.0	0.25	0.05	0.84	0.06	4B

TABLE 3. Subtyping of serotype 4 HRV strains by SPIEM and ELISA, using ST3 (4A)- and VA70 (4B)-specific MAbs

HRV subtype	No. of strains subtyped/no. examined (%)	Subtyping ratio[a]	
		ELISA	SPIEM
4A (ST3-like)	46/59 (78.0)	3.14–33.50	3.20–50.00
4B (VA70-like)	13/59 (22.0)	0.03–0.15	0.05–0.30

[a] Ratio was obtained, for ELISA, by dividing the absorbance in wells reacted with ST3 MAb by that in wells reacted with VA70 MAb and, for SPIEM, by dividing the number of double-shelled rotavirus particles trapped by the ST3 MAb by the number of particles trapped by VA70 MAb.

Subtype 4B strains were shown to cocirculate with subtype 4A HRVs, but so far they have been found only in Italy and South America (Chile) and in stools from children with diarrhea. On the other hand, subtype 4A HRVs were identified in samples from patients with gastroenteritis as well as from healthy infants and young children. No correlation was found between RNA electropherotypes and subtypes of the HRV serotype 4 strains examined (Fig. 3).

DISCUSSION

The results obtained in the present study unequivocally demonstrate that serotype 4 HRVs belong to two distinct subtypes: 4A, including ST3-like strains, which represent about 75% of serotype 4 strains; and 4B, including VA70-like strains, which represent about 25% of the serotype 4 HRVs so far examined. All 59 strains identified as serotype 4 by using animal antisera were definitely subtyped by MAbs. Thus, it seems unlikely that the two subtypes are only minor variants within a well-defined serotype. Furthermore, the two MAbs identified the same subtypes recognized, although less clearly, by cross-absorbed antisera in SPIEM (8). No particular RNA electrophoretic migration pattern was associated with either subtype, in agreement with our previous findings on the electropherotype heterogeniety within HRV serotypes (6). A small but distinct difference in the electrophoretic mobility of unreduced VP7 was consistently observed between ST3 and VA70, but several HRV strains should be analyzed to define its possible relation with subtypes.

Recently, sequence analysis of the VP7 gene from different rotavirus serotypes identified six major regions, A to F, of amino acid divergence (9). Regions B (amino acids 87 to 101) and E (amino acids 208 to 221) are thought to represent the immunodominant NT antigenic site of VP7 glycoprotein. Differences of one amino acid substitution in region B and two substitutions in region E were found between ST3 and VA70 strains. Thus, the immunological

TABLE 4. Serotyping of HRV reference strains by ELISA, using MAbs

MAb to HRV strain (serotype)	ELISA reactivity (A_{492}) with HRV strain (serotype)[a]:				
	DS-1 (2)	Wa (1)	YO (3)	ST3 (4A)	VA70 (4B)
DS-1 (2)	**1.85**	0.04	0.04	0.05	0.03
Wa (1)	0.02	**0.85**	0	0	0
YO (3)	0	0	**>2.00**	0	0
ST3 (4A)	0	0.01	0.02	**0.81**	0.05
VA70 (4B)	0.03	0.02	0.03	0.10	**>2.00**

[a] Homologous reactivity values are given in boldface.
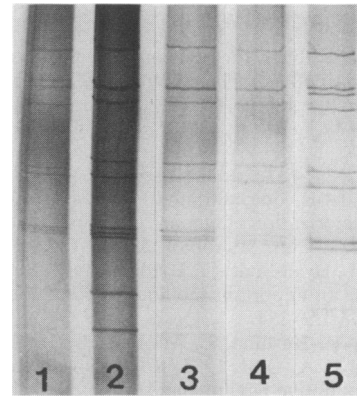

FIG. 3. RNA patterns of serotype 4 HRV strains. Lane 1, ST3 (subtype 4A); lane 2, VA70 (4B); lane 3, PV5249 (4A); lane 4, PV5257 (4B); lane 5, PV11562 (4A).

difference between the two subtypes of HRV serotype 4 might be due to only three amino acid substitutions in the immunodominant NT antigenic site of the VP7 molecule. However, one amino acid substitution at residue 238, where ST3, but not VA70, possesses a potential glycosylation site, might be responsible for the higher electrophoretic mobility of VA70 VP7, as well as for its altered antigenicity (2). Comparative analyses of the VP7 amino acid sequences of several serotype 4 HRV strains would clarify this point.

Initially, cross-NT assays with animal hyperimmune sera showed that the two prototypes of HRV serotype 4 were antigenically different, but that difference was not sufficient to define two distinct serotypes. After cross-absorption of hyperimmune sera to make them type specific (8), in the SPIEM system many, but not all, of the VA70-like (subtype 4B) strains were identified as serotype 4 by ST3 antiserum. Some subtype 4B strains were reactive only with VA70 antiserum. This finding made it necessary to use both ST3 and VA70 animal hyperimmune sera for routine typing of HRVs directly in stool specimens. When MAbs to each of the two subtypes became available, we observed that some cross-reactivity was displayed by ST3 MAb by SPIEM, ELISA, and NT assay, whereas VA70 MAb appeared to be highly subtype specific. On the basis of experimental results obtained by both SPIEM and ELISA, the degree of cross-reactivity possessed by ST3 MAb is high enough to identify as serotype 4 HRV strains of both subtypes, provided that samples are rich in double-shelled virus particles. Conversely, when stool specimens poor in double-shelled virus particles are examined for typing, subtype 4B HRVs cannot be typed unless VA70 (subtype 4B-specific) MAb is used. Thus, the degree of conservation of the immunodominant NT site is likely to be high enough to allow serotyping of both subtypes by ST3 MAbs in specimens in which VP7 of 4B strains is present in large amounts. Otherwise, when stool suspensions poor in VP7 of HRV serotype 4B are examined, it is often necessary to use subtype-specific MAb in order to recognize the variable regions of the subtype 4B NT site.

At the moment we cannot exclude that major variants may exist also within other rotavirus serotypes at the level of both VP7 and VP3 serotype specificities (4).

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