Characterization of the Neutralizing Epitopes of VP7 of the Gottfried Strain of Porcine Rotavirus†

S. Y. KANG,^{1‡} D. A. BENFIELD,² M. GORZIGLIA,³ AND L. J. SAIF^{1*}

Food Animal Health Research Program, Ohio Agricultural Research and Development Center, Ohio State University, Wooster, Ohio 44691¹; Department of Veterinary Science, South Dakota State University, Brookings, South Dakota 57007²; and Laboratory of Infectious Disease, National Institute of Allergy and Infectious Disease, Bethesda, Maryland 20892³

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The neutralization epitopes of the outer capsid protein VP7 of a porcine group A rotavirus were studied by using neutralizing monoclonal antibodies (N-MABs). Six N-MABs which were specific for the VP7 protein of the Gottfried strain of porcine rotavirus (serotype G4) were used for analyzing the antigenic sites of VP7. Three different approaches were used for this analysis: testing the serological reactivity of each N-MAB against different G serotypes of human and animal rotaviruses, analyzing N-MAB-resistant viral antigenic variants, and performing a nucleotide sequence analysis of the VP7 gene of each of the viral antigenic variants generated. From the serological analyses, three different reactivity patterns were recognized by plaque reduction virus neutralization and cell culture immunofluorescence tests. A single MAB (RG36H9) reacted with animal rotavirus serotypes G3 and G4 but not with human serotypes G3 and G4. The MAB 57/8 (D. A. Benfield, E. A. Nelson, and Y. Hoshino, p. 111, in *Abstr. VIIth Internat. Congr. Virol.*, 1987, and E. R. Mackow, R. D. Shaw, S. M. Matsui, P. T. Vo, D. A. Benfield, and H. B. Greenberg, *Virology* 165:511-517, 1988) reacted with animal and human rotavirus serotypes G3 and G4 and also with human serotype G9 and bovine serotype G6. The other four MABs reacted only with the porcine rotavirus serotype G4. The epitope defined by MAB 57/8 and the epitope defined by the other five MABs appeared to be partially overlapping or close to each other, as identified by viral antigenic variant analysis. However, data from nucleotide and deduced amino acid sequence analyses of the VP7 of each of the viral antigenic variants showed that these two epitopes constituted a large, single neutralization domain.

Rotaviruses are established causes of acute gastroenteritis in humans, as well as in other mammals and in avian species (9, 21). At least four G serotypes of group A rotavirus bearing VP7 have been identified among strains isolated from pigs (3, 9, 20). Of the porcine rotaviruses, the Gottfried strain is antigenically related to serotype G4 human rotaviruses (3, 20) whereas the serotype G5 OSU strain is antigenically distinct from known human rotavirus serotypes (3). Other porcine rotavirus isolates have been classified as serotype G3, serotype G11, and mixed serotypes of G3 and G5 (28, 31). Recently, new candidate porcine rotavirus serotypes have been described but not fully characterized (1, 30).

Two outer capsid proteins of group A rotaviruses, VP4 and VP7, are now known to be independent neutralization antigens (15, 16, 18, 19). It has been shown that the serotype-specific antigen involved in virus neutralization is mainly defined by the VP7 glycoprotein (serotype G), which is encoded by RNA segment 7, 8, or 9 (15, 16). By utilizing comparative analysis of nucleotide and deduced amino acid sequences of VP7 genes from different animal and human rotavirus serotypes, nine discrete regions of divergence (VR1 to VR9) were defined (13). Six of these regions were previously defined as divergent regions among serotypes (12, 14, 17).

In the present study, we produced VP7-specific neutraliz-

ing monoclonal antibodies (N-MABs) against the Gottfried strain of porcine rotavirus and used them to characterize the neutralizing epitopes of VP7. The reactivities of these N-MABs with distinct serotypes of animal rotaviruses and symptomatic and asymptomatic human rotaviruses were examined by plaque reduction virus neutralization (PRVN) and cell culture immunofluorescence (CCIF) tests. Viral antigenic variants resistant to the VP7-specific N-MABs were selected for and analyzed. In addition, the nucleotide changes and the deduced amino acid changes within VP7 associated with the antigenic alteration in the variants were examined.

MATERIALS AND METHODS

Viruses and cells. The following pairs of symptomatic and asymptomatic human rotaviruses, each pair serologically distinct, were used for characterizing the N-MABs: Wa and M37 (serotype G1), DS-1 and 1076 (serotype G2), M and McN13 (serotype G3), and VA70 and ST-3 (serotype G4). These human rotavirus strains were provided by Y. Hoshino (National Institutes of Health, Bethesda, Md.). Human rotavirus strain WI61 (serotype G9) (6), supplied by H. F. Clark (The Wistar Institute, Philadelphia, Pa.), was included as a symptomatic human rotavirus. The following were included as animal rotaviruses: simian rotaviruses SA-11 and RRV (serotype G3), obtained from M. K. Estes (Baylor College of Medicine, Houston, Tex.) and Y. Hoshino, respectively, the Gottfried strain of porcine rotavirus (serotype G4); porcine rotavirus strains OSU (serotype G5) and SB-1A (with a serotype G4 VP7 and VP4 like that of OSU); and bovine rotaviruses NCDV Lincoln (serotype G6) and

* Corresponding author.

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‡ Present address: Chungbuk National University, Cheongju, Korea.

B223 (serotype G10, supplied by G. N. Woode [Texas A&M University, College Station, Tex.]). Other serotype G5 porcine rotaviruses (EE and A580) (3, 20), isolated and passaged in our laboratory, were also used. All rotaviruses were plaque purified three times before use.

An African green monkey kidney cell line (MA104) was used throughout this study. The cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U of penicillin, 100 µg of streptomycin, and 25 µg of nystatin per ml).

Production of MAbs. Hybridomas were produced by procedures similar to those used previously in our laboratory (22, 35). Female BALB/c mice, 8 to 10 weeks old, were maintained in plastic isolators under gnotobiotic isolation conditions. Mice maintained in a similar manner were also used for ascites production. The mice were immunized intraperitoneally by using complete Freund's adjuvant with the Gottfried strain of porcine rotavirus, semipurified from the gut contents of an infected gnotobiotic pig. Two weeks later, virus was administered intraperitoneally in incomplete Freund's adjuvant. After an additional 2 weeks, mice were given intravenous boosters of virus in phosphate-buffered saline (PBS) (pH 7.4). Three days after the final booster, spleen cells were fused with SP2/0 myeloma cells by using 50% polyethylene glycol (molecular weight, 3,350; Sigma Chemical Co., St. Louis, Mo.). When hybridomas occupied at least 30% of the well area, culture supernatants were screened for production of neutralizing antibody against the Gottfried strain of porcine rotavirus by a fluorescent focus neutralization (FFN) test. Hybridomas which neutralized the Gottfried strain of porcine rotavirus during the FFN test were cloned at least twice by limiting dilution with mouse thymocyte feeder layers. The immunoglobulin isotype of each MAb was determined by using hybridoma culture fluids in Ouchterlony double immunodiffusion gels with sheep anti-mouse immunoglobulin typing sets (ICN ImmunoBiologicals, Lisle, Ill.).

Pristane-primed BALB/c mice were injected intraperitoneally with cloned hybridoma cells to produce ascitic fluids. The ascitic fluids were filtered (0.45-µm-pore-size filter; Millipore Corp., Bedford, Mass.), heat inactivated (56°C for 30 min), and stored frozen until used in the assays.

FFN test. The FFN test was performed to screen for hybridomas secreting neutralizing antibodies against the Gottfried strain of porcine rotavirus by a modification of methods previously described (22, 35). Briefly, equal volumes of hybridoma culture fluids and virus suspensions containing 10^4 fluorescent focus-forming units per 0.50 ml were mixed and incubated for 1 h at 37°C. MA104 cell monolayers in 96-well plates were inoculated with these mixtures and incubated at 37°C for 18 to 20 h in a 5% CO₂ atmosphere and then fixed with 80% acetone and stained with fluorescein isothiocyanate-conjugated porcine anti-porcine rotavirus (OSU strain) immunoglobulin. Hybridoma supernatants that reduced fluorescent focus-forming units by 60% or more were considered to contain neutralizing antibodies to the Gottfried strain of porcine rotavirus.

CCIF test. The CCIF test was performed to determine the reactivity patterns of N-MAbs against different serotypes of human and animal rotaviruses. Confluent MA104 cell monolayers in 96-well microtiter plates were infected with various human and animal rotaviruses at a multiplicity of infection of 0.1 PFU per cell. After incubation at 37°C for 18 to 20 h, infected cells were rinsed once with PBS and fixed with 80% acetone. Immunoglobulins precipitated from the ascitic fluids with ammonium sulfate and mixed with PBS at a stan-

dard concentration of 10 µg/ml were added to infected, fixed cells and incubated for 1 h at 37°C. After incubation, the plates were washed three times with PBS and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (A, G, and M) (Kirkegaard and Perry, Gaithersburg, Md.) were added to each well at a 1:30 dilution. Following 1 h of incubation at 37°C, the plates were washed twice with PBS. Cells were examined for immunofluorescence with a fluorescence microscope (Olympus IM, Tokyo, Japan), and wells which contained cells fluorescing in the presence of N-MAbs were considered positive. For determination of the titers of the N-MAbs against the homologous Gottfried strain of porcine rotavirus by the CCIF test, serial dilutions of unconcentrated ascites were tested as described above. The titer was expressed as the reciprocal of the highest ascites dilution which resulted in the appearance of fluorescing cells.

PRVN test. The PRVN test was performed in plastic six-well plates containing MA104 cell monolayers to determine titers of the N-MAbs as previously described (35). In brief, serial fourfold dilutions of ascitic fluids were mixed with equal volumes of the viral suspension, containing 60 to 80 PFU/0.1 ml, and incubated at 37°C for 1 h. Then, 0.1-ml volumes of the mixtures were inoculated onto duplicate wells and incubated for an additional 1 h at 37°C. The plates were washed once with serum-free EMEM, and agar medium containing 0.8% noble agar and 67 µg of neutral red and 25 µg of pancreatin (GIBCO Laboratories, Grand Island, N.Y.) per ml (four times the neutralization factor) in EMEM was added. The PRVN titer was expressed as the reciprocal of the highest dilution of ascitic fluid which produced an 80% reduction in the number of plaques compared with the number in wells containing the control virus.

Production of N-MAb-resistant viral antigenic variants. The VP7-specific N-MAbs were used to select viral antigenic variants which were resistant to neutralization. Selection and isolation of the viral antigenic variants were performed by a modification of procedures previously described (33). In brief, 100 µl of serial 10-fold dilutions of the Gottfried strain of porcine rotavirus was mixed with an equal volume of ascitic fluids diluted 1:25 to 1:500 in EMEM and incubated at 37°C for 1 h. MA104 cell monolayers grown in roller tubes were then inoculated with this mixture. After adsorption for 1 h, cells were washed with serum-free EMEM and fed with 2.0 ml of serum-free EMEM containing ascitic fluid diluted 1:500 or 1:2,000 and pancreatin. Infected cells were incubated in a roller tube apparatus and were harvested after 7 days. The growth of each viral antigenic variant was assayed by a CCIF test, and the resistance of each variant to the corresponding N-MAb was confirmed by the FFN test. After an additional two cycles of virus neutralization and multiplication, as described above, the resultant viral antigenic variants were plaque purified at least twice in MA104 cells. Each viral antigenic variant was designated by prefixing the name of the N-MAb used for its selection with the letter v. We picked five plaques for each selecting MAb, and the plaque-purified viral antigenic variants were examined for their patterns of reactivity with the panel of VP7-specific N-MAbs.

Protein specificity of the N-MAbs. The protein specificity of each N-MAb was determined by the PRVN test with the naturally occurring reassortant porcine rotavirus strain SB-1A. The SB-1A strain contains all the protein genes of the Gottfried strain (including the VP7 gene, serotype G4) except for the VP4 gene (serotype P), which is of OSU origin (3, 18, 20).

Nucleotide sequences of viral antigenic variants. The nucle-

TABLE 1. Characterization by isotype, protein specificity, and titer of MAbs produced against the Gottfried strain of porcine rotavirus

MAb ^a	Immunoglobulin isotype	Antibody titer ^b in test	
		PRVN	CCIF
RG36H9	M	270,000	102,400
RG38A3	M	180,000	12,800
RG39C12	G3	64	<100
RG42A6	G3	64	<100
RG43H1	G3	64	<100
57/8	M	350,000	>204,800

^a The protein specificity of the MAbs (from ascites) was VP7, as determined by reaction with an OSU × Gottfried reassortant rotavirus (SB-1A) (VP4: OSU; VP7:Gottfried).

^b PRVN titers against the Gottfried strain of rotavirus are expressed as the reciprocal of the ascites dilution which produced an 80% reduction in plaques compared with the number of plaques in controls. Titers in the CCIF test represent the reciprocal of the ascites dilution which produced immunofluorescence after indirect staining of cell cultures infected with the Gottfried strain of rotavirus with fluorescein-conjugated anti-mouse immunoglobulins.

otide sequences of the VP7 gene of the Gottfried strain of porcine rotavirus and the viral antigenic variants were determined by primer extension analysis (14). Plus-stranded RNA transcripts were prepared from single-shelled rotavirus cores, and dideoxynucleotide sequencing of RNA transcripts was performed by a method described previously (10).

RESULTS

Characterization of MAbs. Both VP4- and VP7-specific N-MAbs were produced during cell fusion, and VP4-specific N-MAbs were characterized previously (22). A panel of six VP7-specific N-MAbs was used in this study, five of which were produced and cloned in our laboratory. The sixth N-MAb, 57/8, was produced previously by Benfield et al. (2). A summary of the properties of these N-MAbs is given in Table 1. All five N-MAbs were shown to be VP7 specific by their neutralization of the Gottfried and SB-1A rotavirus strains (Table 2). The latter virus shares the VP7 G4 serotype

with the Gottfried rotavirus strain but contains an unrelated gene 4 (a VP4 gene like that of OSU rotavirus). A Gottfried VP4-specific N-MAb, RG24B9 (22), was included in the assays as a control. This N-MAb (RG24B9) failed to neutralize rotavirus strain SB-1A.

Reactivity of N-MAbs with various rotavirus strains. (i) **Virus neutralization.** The cross-reactivity of the N-MAbs specific for VP7 of the Gottfried strain with various serotypes of human and animal rotaviruses, determined by the PRVN test, is shown in Table 2. Three distinct patterns of reactivity of the N-MAbs with various human and animal rotavirus serotypes were observed. On the basis of the PRVN results, VP7-specific N-MAbs were divided into three groups (groups I to III) according to their patterns of reactivity with various serotypes of human rotavirus. One N-MAb, categorized as group I (RG36H9), did not neutralize any of the human rotavirus serotypes tested, whereas it reacted with animal rotaviruses of the G3 serotype (SA-11 and RRV). However, another four N-MAbs (RG38A3, RG39C12, RG42A6, and RG43H1), categorized as group II, did not neutralize any of the human and animal rotavirus serotypes examined except for the Gottfried and SB-1A strains (serotype G4). The single N-MAb in group III, 57/8, cross-reacted with symptomatic and asymptomatic human rotavirus serotypes G3 and G4 and with symptomatic human rotavirus serotype G9 (Table 2). This N-MAb also neutralized animal rotavirus serotypes G3 (SA-11 and RRV) and G6 (NCDV). As indicated earlier, all the VP7-specific N-MAbs reacted with SB-1A porcine rotavirus, which shares the VP7 gene with the Gottfried strain of rotavirus and the VP4 gene with the OSU strain of rotavirus (11).

(ii) **CCIF.** The reactivity patterns of the N-MAbs obtained with various serotypes of human and animal rotaviruses, as determined by a CCIF test, are shown in Table 3. On the basis of these results, the N-MAbs were grouped (groups I, II, and III) as described above.

Viral antigenic variant analysis. To map the topographical antigenic sites of VP7, we selected viral antigenic variants from a pool of the Gottfried strain grown in MA104 cells in the presence of each of the N-MAbs. We did not calculate the frequency of viral antigenic variants. However, viral antigenic variants could be readily isolated by three passages

TABLE 2. PRVN titers^a of Gottfried VP-7-specific MAbs against homologous virus, symptomatic and asymptomatic human rotavirus G serotypes, and animal rotavirus G serotypes

Group and MAb (from ascites)	Titer(s) against rotavirus (serotype) ^b										
	Human strain(s)					Animal strain(s)					
	Wa (G1)/ M37 (G1)	DS-1 (G2)/ 1076 (G2)	M (G3)/ McN13 (G3)	VA70 (G4)/ ST-3 (G4)	WI61 (G9)	SA-11 (G3)	RRV (G3)	Gottfried (G4)/ SB-1A (G4)	OSU (G5)	NCDV (G6)	B223 (G10)
I											
RG36H9	<4/<4	<4/<4	<4/<4	<4/<4	<4	3,600	7,000	270,000/270,000	<4	<4	<4
RG38A3	<4/<4	<4/<4	<4/<4	<4/<4	<4	<4	<4	180,000/100,000	<4	<4	<4
II											
RG39C12	<4/<4	<4/<4	<4/4	<4/<4	<4	<4	<4	64/64	<4	<4	<4
RG42A6	<4/<4	<4/<4	<4/4	<4/<4	<4	<4	<4	64/64	<4	<4	<4
RG43H1	<4/<4	<4/<4	<4/4	<4/<4	<4	<4	<4	64/64	<4	<4	<4
III											
57/8	<4/<4	<4/<4	>65,536/300	>65,536/9,300	4,096	>65,536	>65,536	350,000/>65,536	<4	20,000	<4

^a Expressed as the reciprocal of the ascites dilution which produced an 80% reduction in plaques.

^b Human strains are in symptomatic/asymptomatic pairs; animal strains (symptomatic) are paired by serotype only. The human strain listed singly is symptomatic.

TABLE 3. Patterns of reactivity of Gottfried VP7-specific MAbs against various human and animal rotavirus G serotypes in the CCIF test^a

Group and MAb	Pattern of reactivity with rotavirus (serotype) ^b									
	Human strain(s)					Animal strain(s)				
	Wa (G1)/ M37 (G1)	DS-1 (G2)/ 1076 (G2)	M (G3)/ McN13 (G3)	VA70 (G4)/ ST-3 (G4)	WI61 (G9)	SA-11 (G3)/ RRV (G3)	Gottfried (G4)/ SB-1A (G4)	SB-2 (G4)/ SB-5 (G4)	OSU (G5)/ EE (G5)	NCDV (G6)/ B223 (G10)
I										
RG36H9	-/-	-/-	-/-	-/-	-	+/+	+/+	-/-	-/-	-/-
II										
RG38A3	-/-	-/-	-/-	-/-	-	-/-	+/+	-/-	-/-	-/-
RG39C12	-/-	-/-	-/-	-/-	-	-/-	+/+	-/-	-/-	-/-
RG42A6	-/-	-/-	-/-	-/-	-	-/-	+/+	-/-	-/-	-/-
RG43H1	-/-	-/-	-/-	-/-	-	-/-	+/+	-/-	-/-	-/-
III										
57/8	-/-	-/-	+/+	+/+	+	+/+	+/+	-/-	-/-	+/-
RG25A10 ^c	+/+	+/+	+/+	+/+	+	+/+	+/+	+/+	+/+	+/+

^a Ammonium sulfate-precipitated MAbs from ascites were tested at a concentration of 10 µg/ml against MA104 cells infected with various rotaviruses, incubated in 96-well tissue culture plates. +, presence of specific fluorescing cells; -, absence of specific fluorescing cells.

^b Human strains are in symptomatic/asymptomatic pairs; animal strains (symptomatic) are paired by serotype only. The human strain listed singly is symptomatic.

^c VP6-specific MAb used as a control.

of the virus in the presence of the N-MAb in roller tube cell cultures prior to plaque purification (data not shown).

We attempted to isolate different kinds of viral antigenic variants against each N-MAb, on the basis of distinctive plaque characteristics (size, opacity, etc.) which might cause different neutralization reactivity patterns with the panel of VP7-specific N-MABs. We obtained eight viral antigenic variants by using N-MABs directed against the Gottfried strain of porcine rotavirus. The patterns of reactivity between these eight viral antigenic variants and the panel of VP7-specific N-MABs, determined by cross-neutralization tests, are summarized in Fig. 1. Three distinct reactivity patterns were observed. RG36H9, categorized as group I, selected one viral antigenic variant, v-RG36H9, which was resistant to neutralization not only by the homologous N-MAb used for its selection but also by four heterologous N-MABs (RG38A3, RG39C12, RG42A6, and RG43H1). However, v-RG36H9 was sensitive to neutralization by N-MAb 57/8. Two N-MABs (RG42A6 and G43H1) categorized as group II each selected two distinct viral antigenic variants, v-RG42A6/A and v-RG42A6/B for RG42A6 and v-RG43H1/A and v-RG43H1/B for G43H1. They behaved differently with the panel of VP7-specific N-MABs. For example, v-RG42A6/A was sensitive to N-MAb RG38A3, whereas v-RG42A6/B was resistant to neutralization by the same N-MAb. However, two N-MABs (RG39C12 and RG38A3), also categorized as group II, selected viral antigenic variants v-RG39C12 and v-RG38A3, respectively, and their reactivity patterns were the same as that of v-RG36H9. The group III N-MAb, 57/8, selected one viral antigenic variant, and its reactivity pattern was the same as that of v-RG43H1/A. Only v-RG43H1/A and the homologous variant (v-57/8) were resistant to neutralization by N-MAb 57/8. v-RG42A6/A was resistant to neutralization by the homologous N-MAb RG42A6 and by heterologous N-MABs RG36H9 and RG39C12, but it was sensitive to RG38A3.

Nucleotide sequence analysis of antigenic variants. To map the antigenic sites on VP7 of the Gottfried strain, we sequenced the VP7 gene of the parent Gottfried strain and that of its antigenic variants which were resistant to the

N-MABs used for their selection. Table 4 shows the nucleotide sequence of the VP7 gene and the deduced amino acid sequence changes in the viral antigenic variants. Only single nucleotide changes were found on the entire VP7 gene of each viral antigenic variant.

		N-MABs							
		36H9	39C12	42A6	43H1	38A3	57/8	24B9	N1016
V A R I A N T S	36H9	■	■	■	■	■	□	□	□
	39C12	■	■	■	■	■	□	□	□
	42A6/A	■	■	■	■	□	□	□	□
	42A6/B	■	■	■	■	■	□	□	□
	43H1/A	■	■	■	■	■	■	□	□
	43H1/B	■	■	■	■	■	■	□	□
	38A3	■	■	■	■	■	■	□	□
	57/8	■	■	■	■	■	■	□	□
	24B9	□	□	□	□	□	□	■	□
	Gott	□	□	□	□	□	□	□	□

FIG. 1. Antigenic sites on VP7 of the Gottfried strain of porcine rotavirus. A panel of VP7-specific N-MABs was tested for neutralization of the parent virus (Gott) and the N-MAB-resistant viral antigenic variants by the FFN test. The viral antigenic variants were designated as resistant (■) or sensitive (□) to neutralization with each N-MAb. The criterion for resistance was that the titer of the N-MAb against a given variant be at least 32-fold lower than the titer against the parent virus. Viral antigenic variant v-RG24B9, which is resistant to VP4-specific N-MAb RG24B9, was included as a control. N1016 represents porcine hyperimmune anti-Gottfried porcine rotavirus serum.

TABLE 4. Nucleotide and amino acid sequence changes found in the viral antigenic variants selected with VP7-specific N-MAbs

Antigenic variant	Selecting N-MAb	Codon change (position)	Amino acid change (position)
v-RG36H9	RG36H9	AAT to GAT (328)	Asn to Asp (94)
v-RG38A3	RG38A3	AAT to GAT (328)	Asn to Asp (94)
v-RG39C12	RG39C12	AAT to GAT (328)	Asn to Asp (94)
v-RG42A6/A	RG42A6	AAT to AGT (335)	Asn to Ser (96)
v-RG42A6/B	RG42A6	AAT to ACT (335)	Asn to Thr (96)
v-RG43H1/A	RG43H1	AAT to GAT (335)	Asn to Asp (96)
v-RG43H1/B	RG43H1	AAT to GAT (335)	Asn to Asp (96)
v-57/8	57/8	AAT to GAT (328)	Asn to Asp (94)

DISCUSSION

N-MAbs directed against the VP7 of strains SA-11 and RRV, three serotypes of porcine rotaviruses, and human rotavirus strains have been described previously (4, 7, 16, 27, 32, 34). We repeatedly failed to determine the viral protein specificities of our VP7-specific MABs by Western blot (immunoblot) analysis (data not shown), confirming other reports that many VP7-specific N-MAbs do not react with denatured VP7 in a Western blot or by immunoprecipitation (7, 11, 34). However, each of our N-MAbs was confirmed as VP7 specific by its neutralization of the naturally occurring reassortant porcine rotavirus strain SB-1A, which contains an unrelated gene 4 (a VP4 gene) from a serotype G5 (OSU strain) rotavirus but gene 9 (a VP7 gene) from a serotype G4 (Gottfried strain) rotavirus (19).

Morita et al. (26) also determined the protein specificities of N-MAbs using gene reassortants. However, protein specificity determined only by using reassortant viruses should be analyzed carefully, on the basis of the results obtained by Chen et al. (5), who showed that VP4-specific MABs could react in reassortant studies as if they recognized VP7 because of steric interactions between VP4 and VP7. In our experiment, all the N-MAbs tested neutralized the SB-1A reassortant rotavirus (Table 2). VP4-specific N-MAbs to the Gottfried strain of rotavirus were produced and characterized previously, and protein specificity was determined by an immunoprecipitation test. None of the VP4-specific N-MAbs neutralized SB-1A, further confirming their VP4 specificity (22).

N-MAbs and their viral antigenic variants are useful for analyzing the antigenic determinants of viral proteins which are involved in neutralization (8, 24–26, 32, 33). The genetic changes sustained by each viral antigenic variant which is resistant to the selecting N-MAb should also confer resistance to other N-MAbs which recognize similar antigenic determinants.

Although differences in the serological reactivity patterns suggested three distinct groups of N-MAbs, the viral antigenic variant analyses confirmed nonreciprocal reactions indirectly linking at least three neutralization epitopes within a single domain (Fig. 1). Competitive binding experiments with our N-MAbs will be required to elucidate the structural relationship between the identified epitopes. Viral antigenic variant analysis can create a relative map of the antigenic topography of the viral surface and can identify the amino acids that directly or indirectly affect neutralization epitopes, but it cannot identify the exact location of neutralization epitopes. When the nucleotide sequence of the VP7 gene of each viral antigenic variant was compared with that of the VP7 gene of the parent virus, only a single nucleotide change was found on the entire VP7 gene in each viral

antigenic variant. All viral antigenic variants selected with N-MAbs had an amino acid change in region A (amino acids 87 to 101). The VP7-specific N-MAbs did not select any viral antigenic variants that had a mutation in region B (amino acids 145 to 150) or region C (amino acids 211 to 213). Except for this difference, our observations with VP7-specific N-MAbs against the Gottfried strain of porcine rotavirus (serotype G4) are similar to those previously reported for other rotavirus serotypes (25, 26, 32, 33).

On the basis of VP7 gene sequence analysis of neutralization-resistant antigenic variants selected with serotype G3-specific homotypic N-MAbs against SA-11 rotavirus, Dyall-Smith et al. (8) identified three distinct regions (A, B, and C) related to neutralization, two of which (A and C) appeared to be close together on the native VP7 glycoprotein. Also, the same large neutralization domain with several interrelated epitopes on VP7 was defined by several investigators using different serotypes of rotaviruses (26, 32, 33). These findings suggest that the immunodominant antigenic sites for neutralization on VP7 resides are at the same positions in rotaviruses which are of different serotypes as well as from different species.

Recently, however, Kobayashi et al. (23) identified a new neutralization epitope (S-2) on VP7, which is distinct from the previously recognized ones. There are several reports describing heterotypic epitopes on rotavirus VP7 that induce cross-neutralizing antibodies. Dyall-Smith et al. (8) reported that there is a close spatial relationship between the A and C regions; in their study, antigenic variants in both regions were selected with the same N-MAb. Mackow et al. (25) demonstrated that even though heterotypic N-MAb 57/8 binds to an antigenic site made up of both the A and C regions, the variants selected with this N-MAb had a mutation at amino acid 94 in the A region, the same amino acid location selected with homotypic N-MAbs. They proposed the hypothesis that VP7-specific heterotypic N-MAbs recognize epitopes composed of A and C regions which are conformation dependent. Taniguchi et al. (33) showed that one heterotypic N-MAb specific for VP7 of serotypes G1, G3, and G4 selected variants with mutations at the same position in region A (amino acid 96, 98, or 99). Our experiments showed similar results. Viral antigenic variants selected with the heterotypic N-MAbs RG36H9 (which neutralizes serotypes G3 and G4) and 57/8 (which neutralizes serotypes G3, G4, G6, and G9) had a mutation within the A region (amino acid 94, Asn → Asp), and serotype-specific neutralization epitopes recognized by N-MAbs RG38A3, RG39C12, RG42A6, and RG43H1 mapped in the same region (amino acid 94 or 96, Asn → Asp or Asn → Ser or Thr). Although the homologous PRVN titers of three of the group II MABs (RG39C12, RG42A6, and RG43H1) were low (titers of 64), they were effective in inducing escape mutations in this known antigenic site and they displayed reactivity patterns similar to that of a fourth MAB in group II (RG38A3) with a high PRVN titer (180,000). Because we did not recover viral antigenic variants with mutations in region C (amino acids 211 to 213), we could not test and confirm the conclusions of Mackow et al. (25) regarding heterotypic neutralization epitopes composed of A and C regions. More VP7-specific N-MAbs are needed to resolve this question.

The role of VP7 in heterotypic immunity is unclear. At first, VP4 appeared to be responsible for the induction of heterotypic immunity (18, 19, 29). Several VP4-specific N-MAbs were cloned and shown to be cross-reactive with different serotypes of human and animal rotaviruses (22). However, we and others produced VP7-specific N-MAbs

that neutralized different serotypes of human and animal rotaviruses (2, 25, 33), suggesting that VP7 also plays a role in heterotypic immunity to rotaviruses.

In summary, we selected viral antigenic variants to be tested against a panel of VP7-specific N-MAbs, and three distinct patterns of reactivity between these variants and the N-MAbs were observed. The N-MAbs showed three different patterns of reactivity against various G serotypes of human and animal rotaviruses in PRVN and CCIF tests. Nevertheless, viral antigenic variants were selected at two closely spaced amino acids (Asn-94 and -96), resulting in a change to one of three other amino acids (Asp, Ser, or Thr). These results imply that the actual binding sites of the N-MAbs are not identical.

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