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Single Point Mutations May Affect the Serotype Reactivity of Serotype G11 Porcine Rotavirus Strains: a Widening Spectrum?

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A panel of single and double neutralization-resistant escape mutants of serotype G11 porcine rotavirus strains A253 and YM, selected with G11 monotype- and serotype-specific neutralizing monoclonal antibodies (MAbs) to VP7, was tested in neutralization assays with hyperimmune sera raised against rotavirus strains of different serotypes. Escape mutants with an amino acid substitution in antigenic region A (amino acids [aa] 87 to 101) resulting in a residue identical or chemically similar to those present at the same positions in serotype G3 strains, at positions 87 for strain A253 and 96 for strain YM, were significantly more sensitive than the parental strains to neutralization with sera against some serotype G3 strains. Also, one YM antigenic variant (YM-5E6.1) acquired reactivity by enzyme-linked immunosorbent assay with MAbs 159, 57/8, and YO-1E2, which react with G3 strains, but not with the serotype G11 parental strain YM. Cross-adsorption studies suggested that the observed cross-neutralization by the G3-specific sera was due to the sera containing antibodies reactive with the parental strain plus antibodies reactive with the epitope(s) on the antigenic variant that mimick the serotype G3 specific one(s). Moreover, antibodies reactive with antigenic region F (aa 235 to 242) of VP7 might also be involved since cross-reactivity to serotype G3 was decreased in double mutants carrying an additional mutation, which creates a potential glycosylation site at position 238. Thus, single point mutations can affect the serotype reactivity of G11 porcine rotavirus strains with both monoclonal and polyclonal antibodies and may explain the origin of rotavirus strains with dual serotype specificity based on sequence divergence of VP7.

Group A rotaviruses are the most common etiological agents of viral acute gastroenteritis in humans and in several animal species, and a worldwide effort is being made to design an effective vaccination strategy. Rotaviruses belong to the *Reoviridae* family and are composed of three concentric protein layers surrounding the 11 segments of double-stranded RNA (16). Two proteins present on the outer capsid of rotaviruses, VP4 and VP7, have been shown to independently elicit antibodies capable of neutralizing infectivity and induce protective immunity (19, 33). Fourteen G serotypes based on VP7 specificity have been defined by cross-neutralization tests with hyperimmune sera (16). Among porcine rotaviruses, strains representative of serotypes G1, G3, G4, G5, G10, and G11 have been described (2, 6, 7, 9, 21, 29, 34, 35). The G serotype specificity is the result of a high degree of sequence divergence in hypervariable regions of the VP7 gene that are nevertheless conserved within a serotype; therefore, the serotype specificity is determined by a consensus sequence of amino acids at several sites (17). Studies with monoclonal antibodies (MAbs) to VP7 have been instrumental in understanding the antigenic properties of this protein. Studies with MAb neutralization-resistant escape mutants have identified regions A (amino acids [aa] 87 to 101), B (aa 141 to 151), C (aa 208 to 224), D (aa 291), E (aa 189 to 191), and F (aa 235 to 242) on VP7 that are involved in neutralization (7, 10, 12, 14, 15, 23, 24, 27, 32).

The antigenic variation seen in rotavirus appears to have evolved in a fashion similar to that of the hemagglutinin and neuraminidase genes of influenza A viruses: antigenic shift (resulting from gene reassortment) and antigenic drift (resulting from the successive accumulation of mutations) (9, 17, 30–32, 39). Available data linking sequence divergence of VP7 within a given rotavirus serotype to antigenic drift are limited. Nishikawa et al. (32) reported an evolutionary analysis of the VP7 gene of serotype G3 rotaviruses from several species in which the different reactivities of three anti-VP7 MAbs with 27 strains were consistent with the occurrence of antigenic variation among G3 strains. Similarly, Ciarlet et al. (9) reported sequence divergence among porcine strains of serotypes G3 and G5 that was associated with different reactivities with MAbs produced against some of those strains (10). In Australia, a porcine strain with dual (G3/G5) serotype specificity and another porcine G3 strain that showed marked epitope variation from other porcine G3 isolates have been described; their origin was attributed to the accumulation of sequential mutations in the VP7 gene (30, 31). Since these data are limited to rotavirus serotypes G3 and G5, the occurrence of antigenic drift based on sequence divergence of VP7 should be confirmed for other rotavirus serotypes.

In our previous study, we reported the identification of neutralization epitopes on VP7 of serotype G11 porcine strains with neutralization-resistant variants selected with neutralizing G11 monotype- and serotype-specific MAbs (7). In this study, we report single and double VP7 neutralization-resistant variants of serotype G11 porcine rotavirus strains differing in a single amino acid substitution that exhibit dual (G11/G3) serotype cross-reactivity with both monoclonal and some polyclonal antibodies. This work provides further data on sequence divergence of VP7 to antigenic drift and may explain the origin of rotaviruses with dual serotype specificity.

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TABLE 1. Serological analysis of double antigenic variants with VP7-specific MAbs

Antigenic variant strain	Neutralizing titer[a]					
	6E10 (A, 87)	3C8 (A, 87)	7F10 (A, 87)	8E8 (A, 87)	5E6 (A, 87)	8D10 (C, 223)
A253-3C8/8D10	<100	<100	<100	<100	<100	<100
A253-8E8/8D10	<100	<100	<100	<100	<100	<100
A253-8D10/6E10	<100	<100	<100	400	400	<100
A253-8D10/7F10	<100	<100	<100	<100	<100	<100
A253-8D10/8E8	<100	<100	<100	<100	<100	<100
A253[b]	51,200	3,200	51,200	102,400	102,400	102,400
A253-3C8[b]	<100	<100	<100	<100	1,600	12,800
A253-8E8[b]	<100	<100	<100	<100	800	51,200
A253-8D10[b]	25,600	6,400	25,600	51,200	102,400	<100

[a] Expressed as the reciprocal of the highest dilution of ascitic fluid that reduced the fluorescent focus count by more than 66%. VP7 variable region and position of amino acid change of single neutralization-resistant escape mutants (7) with each MAb are shown in parentheses.
[b] Data were extracted from a previously published article (7) for comparison.

MATERIALS AND METHODS

Viruses and antisera. Porcine rotavirus strain YM (P9[7], G11) and its antiserum were obtained from C. Arias (Departamento de Biología Molecular, Cuernavaca, Mexico). Bovine rotavirus strain B223 (P8[11], G10), equine rotavirus strains L338 (P12[18], G13) and FI23 (P11[12], G14), and their antisera (except that for B223) were obtained from D. R. Snodgrass and P. Iša (Moredun Research Institute, Edinburgh, Scotland, United Kingdom). Other rotavirus strains used in this study were Wa (P1A[8], G1), DS-1 (P1B[4], G2), P (P1A[8], G3), RRV (P5B[3], G3), SA11 (P[2], G2), A411 (P9[7], G3), A131 (P9[7], G3), A138 (P9[7], G3), LCA843 (P[13], G3), CU-1 (P5A[3], G3), FI14 (P11[12], G3), H-2 (P11[12], G3), Gottfried (P2A[6], G4), OSU (P9[7], G5), A34 (P[?], G5), A46 (P[13], G5), C134 (P9[7], G5), NCDV-Lincoln (P6[1], G6), 69M (P4[10], G8), WI61 (P1A[8], G9), A253 (P9[7], G11), L26 (P1B[4], G12), and FR4 (P[12], G14). The viruses were propagated in MA104 cells with trypsin as described previously (7). Hyperimmune sera were raised in guinea pigs and rabbits from rotavirus-free colonies.

N-MAbs. Monotype- and serotype-specific neutralizing MAbs (N-MAbs) 6E10, 3C8, 7F10, 8E8, 5E6, and 8D10 reactive with VP7 of porcine G11 rotavirus were derived and characterized previously (Tables 1 and 2) (7). MAb 7D2 (reactive with a subset of porcine G3 strains and with most G5 strains), MAb 4E3 (reactive with another subset of G3 porcine strains), and MAbs 1C3 and 1C10 (G5 serotype and G5 monotype specific, respectively) were described elsewhere (10). Cross-reactive MAb 57/8 (recognizes serotypes G3, G4, G6, G9, G10, and G14 by enzyme-linked immunosorbent assay [ELISA]) was obtained from D. Benfield (South Dakota State University, Brookings) (4, 8, 10, 28). MAb 159 (recognizes serotypes G3, G10, and G13 by ELISA) was kindly provided by H. B. Greenberg (Palo Alto VA Medical Center, Palo Alto, Calif.) (4, 18, 36). MAbs KU-4, S2-2G10, and ST3-2G7, specific for serotypes G1, G2, and G4, respectively, and MAb YO-1E2 (recognizes serotypes G3 and G5 by ELISA) were commercially obtained from the kit Serotec Rota-MA (Serotec Laboratory, Ebetsu, Japan) (11, 37).

TABLE 2. Nucleotide and amino acid sequence changes of double antigenic variants[a] with VP7-specific MAbs

Parental variant[b]/ selecting MAb	Codon change	Amino acid change (position)	VP7 region
A253-3C8/8D10	AGA→ATA	Arg→Ile (87)	A[b]
	GAT→AAT	Asp→Asn (238)[c]	F
A253-8E8/8D10	AGA→ACA	Arg→Thr (87)	A[b]
	GAT→AAT	Asp→Asn (238)[c]	F
A253-8D10/6E10	AAA→GAA	Lys→Glu (223)	C[b]
	ACA→ATA	Thr→Ile (91)	A
A253-8D10/7F10	AAA→GAA	Lys→Glu (223)	C[b]
	AGA→ACA	Arg→Thr (87)	A
A253-8D10/8E8	AAA→GAA	Lys→Glu (223)	C[b]
	AGA→GGA	Arg→Gly (87)	A

[a] Nucleotide and amino acid sequences of double antigenic variants were compared with that of parent strain A253 and previously isolated single antigenic mutants (7).
[b] Nucleotide and amino acid sequences of the parental variants of strain A253 were reported previously (7).
[c] Mutation results in a different variable region, F (aa 235 to 242), and in formation of a new potential glycosylation site.

Selection of double MAb neutralization escape mutants. The selection and characterization of single escape mutants of G11 porcine rotavirus strains A253 and YM were previously described (7). Each double MAb neutralization escape mutant of strain A253 was derived from an amplified clone of the corresponding single escape mutant virus as described previously (7). From each selection, two to three viral plaques were amplified prior to all cross-neutralization tests performed by focus fluorescent neutralization assay (FFNA) between the N-MAbs and the double mutants. Each double antigenic variant is designated by the name of the first antigenic variant (7) followed by the name of the second selecting N-MAb used. Double antigenic mutants generated by using A253-6E10 or A253-5E6 as the parental strain and MAb 8D10 as the selecting MAb were attempted several times, but no neutralization-resistant variants could be obtained. All double escape mutants exhibited an electrophoretic profile identical to those of both the parent strain A253 and single antigenic variant used to select them, as visualized by polyacrylamide gel electrophoresis and silver staining.

In vitro transcription and dideoxynucleotide sequencing. The amino acid substitutions responsible for resistance to N-MAbs in double antigenic variants were identified by dideoxynucleotide sequencing of single-stranded RNA transcripts, prepared from purified single-shelled particles as described previously (7).

FFNA and ELISA. Neutralization tests were performed as described previously (7). A distinct serotype is defined as a reciprocal 20-fold or greater difference in neutralization titer when a new strain is tested against prototype strains representing known G serotypes (16). The ELISA was carried out with MAb (ascitic fluid) as the capture antibody and rabbit antiserum (against the A253 strain) as the detector antibody (7). A similar concentration of infectious virus particles (10^6 focus-forming units [FFU]/ml) was used as an approximate estimate of the concentration of triple-layered particles. Readings of >0.2 were considered positive reactions, after zero calibration was performed by using the lowest value of wells with an irrelevant MAb as the capture antibody.

Immunoadsorption of the guinea pig anti-RRV hyperimmune serum 32251. Diluted (1/800) serum was incubated for 24 h at 4°C with an excess of infectious viral particles (1×10^6 to 9×10^8 FFU/ml) of each of rotavirus strains RRV (G3) and A253 (G11) and antigenic variant A253-5E6. The adsorbed serum was centrifuged in a Beckman SW41 rotor for 5 h at 4°C at 35,000 rpm (100,000 × *g*). Supernatants were collected, and each was treated with 10 mM EDTA for 12 to 18 h at 4°C to eliminate any infectious viral particles still present. Parallel control serum was also treated with the chelating agent. Control and adsorbed sera was diluted 1:2 prior to all cross-neutralization tests.

RESULTS

Neutralization patterns of antigenically selected variants with hyperimmune sera against rotavirus strains of different serotypes. Antigenic variants, their parental strains, and standard rotavirus strains were tested by neutralization with hyperimmune sera against different rotavirus serotypes (Fig. 1). All standard antisera used in this study had no reactivity with strains belonging to a G serotype other than the one to which they were raised (data not shown), with the exception of one anti-RRV serum (32251) that efficiently neutralized RRV and other G3 strains and one of the serotype G14 equine strains (FI23) (Fig. 1). With the exception of variant YM-5E6.1, all

Virus Strain (P, G type)	Amino Acid Sequence of Indicated Antigenic Hypervariable Regions and the Corresponding Amino Acid Positions			Reactivity with Hyperimmune Sera to strain (P, G type):										
	A (aa 87-101)	C (aa 208-224)	F (aa 235-242)	A253 P9[7], G11 (6,400)	A253-5E6 P9[7], G11 (6,400)	YM P9[7], G11 (3,200)	RRV^{32251} P5B[3], G3 (51,200)	RRV^{13610} P5B[3], G3 (6,400)	H-2 P11[12], G3 (12,800)	FI14 P11[12], G3 (6,400)	CU-1 P5A[3], G3 (51,200)	K9 P5A[3], G3 (12,800)	P P1A[8], G3 (19,200)	A411 P9[7], G3 (12,800)	FI23 P11[12], G14 (6,400)	FR4 P[12], G14 (12,800)
A253 (P9[7], G11)	R E A A T Q I A D D K W K D T	L T T D P T T F E E V A S T E K L	H K L D V T T A	1	2	4	32	32	>64	32	>64	>64	>64	32	>64	>64
A253-3C8	I			2	1	2	2	8	8	4	8	32	>64	32	>64	>64
A253-3C8/8D10	I		N	4	2	4	8	32	64	32	>64	64	>64	32	>64	64
A253-6E10	T			2	1	4	2	8	8	8	8	32	>64	32	>64	>64
A253-8E8	T			2	1	2	2	8	8	4	8	32	>64	32	>64	>64
A253-8E8/8D10	T		N	4	4	4	8	32	64	32	>64	64	>64	32	>64	32
A253-5E6	T			1	1	2	4	8	8	4	8	32	>64	32	>64	>64
A253-7F10	G			4	4	8	32	32	>64	32	64	64	>64	32	>64	>64
A253-8D10		E		1	1	4	32	32	>64	32	32	64	>64	32	>64	>64
A253-8D10/6E10	I	E		2	2	4	32	32	64	32	32	64	>64	32	>64	>64
A253-8D10/7F10	T	E		2	1	4	4	8	8	8	8	32	>64	32	>64	>64
A253-8D10/8E8	G	E		1	4	4	32	32	64	32	>64	>64	>64	64	>64	>64
YM (P9[7], G11)	H	A		2	2	1	32	32	>64	>64	32	>64	>64	>64	>64	>64
YM-5E6.1	H N	A		8	8	16	2	8	8	8	4	32	32	32	>64	>64
YM-5E6.5	H A	A		2	2	1	32	32	>64	>64	32	>64	>64	32	>64	>64
YM-8E8	H A E	A		2	2	1	32	32	>64	>64	32	>64	>64	32	>64	>64
RRV (P5B[3], G3)	T E N S	T A T A	S	>64	32	32	1	1	4	4	8	8	1	2	32	>64
P (P1A[8], G3)	T E N S	T N T A	N N	>64	64	nd	1	4	4	4	4	8	1	2	32	64
H-2 (P11[12], G3)	T E N S	V A I T A		>64	64	nd	2	4	1	1	2	8	4	8	32	32
FI14 (P11[12], G3)	T V A E N S	T T A	S	>64	64	nd	1	4	1	1	4	4	8	8	32	32
CU-1 (P5A[3], G3)	T E N S	V S T	T	>64	64	nd	2	4	2	2	1	1	4	8	24	64
A411 (P9[7], G3)	T E N S	T N I T N	S N	>64	64	>32	1	4	4	4	4	4	4	1	>64	>64
FI23 (P11[12], G14)	T A S S	N V E T S	V E C T	32	32	nd	8	32	24	24	32	64	64	>64	1	1
FR4 (P11[12], G14)	T A S S	N V A T S	I N T	32	32	nd	24	64	32	32	64	64	64	>64	2	1

FIG. 1. Cross-neutralization between rotavirus strains and antigenically selected variants of porcine strains A253 and YM (G11) with hyperimmune sera directed against different rotavirus serotypes. Neutralization titer is defined as the highest serum dilution which inhibits >66% of infectious foci, as measured by immunofluorescence. Shaded areas show <20-fold differences in neutralization titers with heterologous sera. Reactivity is expressed as the ratio of the reciprocal values of neutralization titers of homologous strain (shown in parentheses) to the indicated rotavirus strains. A decrease in numerical value represents an increase in susceptibility to neutralization. Cross-neutralization tests between the antigenic variants and the serotype G3 antisera were performed at least five times, and no more than a twofold variation was observed. The sequences of strains A253, A253-3C8, A253-6E10, A253-8E8, A253-5E6, A253-7F10, A253-8D10, YM-5E6.1, YM-5E6.5, and YM-8E8 (7); YM (35); RRV, P, H-2, FI14, and CU-1 (32); A411 (9); FI23 (3); and FR4 (8) have been published.

antigenic variants were neutralized with sera directed to the parental strain. Inversely, variant YM-5E6.1 was 16 times more susceptible than the parental strain to neutralization with a hyperimmune serum raised in guinea pigs against G3 simian strain RRV (32251). Also, four A253 variants (A253-3C8, A253-6E10, A253-8E8, and A253-5E6) showed 8- to 16-fold increased susceptibility to neutralization with the same anti-RRV serum. Our results suggest that the positions and nature of the amino acid substitutions (Fig. 1) of these escape mutants (Asn at position 96 in YM-5E6.1 and Thr or Ile, but not Gly, at position 87 in A253 variants) were critical in defining new epitopes, probably similar to the immunodominant neutralizing ones present on VP7 of RRV (G3). Neither mutation at position 91 (variants YM-5E6.5 and YM-8E8) or 223 (variant A253-8D10) nor a mutation at position 87 that substituted Arg for Gly (variant A253-7F10) resulted in any increase in susceptibility of anti-RRV serum 32251 (Fig. 1).

To rule out that the neutralization of the escape mutants was due to the hyperimmune serum used (RRV serum 32251), the reactivities of another RRV serum (13610) and several other sera raised to different G3 rotavirus strains were evaluated with all of the variants. The same four A253 antigenic variants and variant YM-5E6.1 were four to eight times more susceptible to neutralization by hyperimmune sera raised in guinea pigs against serotype G3 equine strains H-2 and FI14 and against canine strain CU-1 and the other serum against RRV (serum 13610). However, these mutants were not neutralized with G3-specific hyperimmune sera raised in guinea pigs and rabbits against human strain P and canine strain K9 and against porcine strain A411, respectively (Fig. 1). Also, guinea pig sera against the lapine strain ALA (G3) and the murine strain EB (G3) and rabbit antisera raised against porcine strains A131 (G3) and A138 (G3) (9) failed to neutralized any of these antigenic variants (data not shown). Antisera to rotavirus strains Wa (G1), C60 (G1), C95 (G1), DS-1 (G2), Gottfried (G4), OSU (G5), A46 (G5), C134 (G5), NCDV-Lincoln (G6), 69M (G8), WI61 (G9), B223 (G10), and L338 (G13) did not neutralize either G11 strains or any escape mutants (data not shown). Both serotype G11 rotavirus strains and all antigenic variants were equally neutralized by antiserum to A253-5E6, and no significant increase in susceptibility to selected serotype G3 rotavirus strains was observed. We attempted several times to make antiserum against YM-5E6.1 to examine whether the antiserum would neutralize G3 or G11 rotavirus strains, but all the rabbits died due to rotavirus-unrelated causes before any serum could be collected.

Neutralization patterns of double antigenic variants and identification of amino acid substitutions on VP7. To determine whether additional mutations in the serotype G11 single antigenic variants would increase the neutralization ability of any serum raised against a serotype G3 strain and decrease reactivity to serotype G11 antiserum raised against strain A253, double antigenic variants were selected. Variant A253-8D10 was used to select three double mutants, each one with MAbs 6E10, 7F10, and 8E8, and variants A253-3C8 and A253-8E8 were used as parental viruses to MAb 8D10. Mutants were considered to be resistant to a given N-MAb when the neutralization titer for the mutant was $\leq$32-fold compared to the titer with the parental strain. All double escape mutants escaped neutralization with the MAb used to select them and retained resistance to neutralization by the MAb initially used to select the first variant (Table 1).

Double antigenic variants selected for this study were found to possess amino acid changes at either position 87 or position 91 (region A) and position 238 (region F) when their complete VP7 sequences were compared with those of the single mutant parent viruses (Fig. 1; Table 2). Double mutants A253-8D10/7F10 and A253-8D10/8E8 each showed a mutation at aa 87 (Arg to Thr and Arg to Gly, respectively), while A253-8D10/6E10 showed a mutation at aa 91 (Thr to Ile). Residues at positions 87 and 91 of region A are part of the epitope(s) recognized by MAbs 7F10, 8E8, and 6E10 (7). The mutations in double variants A253-3C8/8D10 and A253-8E8/8D10 were mapped at position 238 (Asp to Asn, region F), different from that identified in variant A253-8D10 (position 223, region C) (7). Therefore, the epitope recognized by MAb 8D10 overlaps antigenic regions C and F. The change of Asp to Asn at position 238 resulted in a new potential glycosylation site which is utilized as shown by a decreased electrophoretic mobility of the VP7 of both double mutants compared with that of the parent mutants (data not shown).

When the double antigenic variants were tested against the panel of hyperimmune sera, a substitution of Arg for Thr at position 87 of a mutant carrying an amino acid substitution at position 223 (variant A253-8D10/7F10) was again involved in the increased susceptibility to neutralization (four- to eight-fold) of the mutant with the same G3 antisera (Fig. 1). An amino acid substitution of a Arg for Gly at position 87 in the same mutant originally carrying an amino acid substitution at position 223 (A253-8D10/8E8) did not result in any increase in susceptibility to any G3 antisera, suggesting that the nature of the substitution in position 87 of the G11 VP7 is critical. The addition of a mutation potentially causing a new glycosylation site at position 238 (Asp to Asn) in the mutants that carried a Thr or Ile at position 87, variants A253-3C8/8D10 and A253-8E8/8D10, eliminated the reactivity with all G3 hyperimmune sera except with RRV serum 32251, although the susceptibility was fourfold lower in comparison with the parental single mutant strains, A253-3C8 and A253-8E8 (Fig. 1). These results suggest that antibodies reactive to antigenic region F (aa 235 to 242) of VP7 might be involved in neutralization since cross-reactivity with G3 antisera was decreased in the double mutants carrying a mutation at position 238. Nevertheless, the mutation at position 238 in these double mutants blocked neutralization only by hyperimmune sera to G3 strains, not that by hyperimmune sera to G11 strains or to the variant A253-5E6. The double mutants were not neutralized by any antisera to the other standard rotavirus strains representative of serotypes G1, G2, G4 to G6, G8 to G10, G13, and G14 (data not shown).

Reactivity patterns of all antigenic variants with G11 and G3 VP7 N-MAbs by ELISA. Single antigenic mutants were previously evaluated by ELISA with VP7-specific MAbs to G11 porcine rotavirus strains (7). For this study, double antigenic variants were evaluated with the same set of serotype G11-specific MAbs, and both single and double antigenic variants were evaluated with MAbs produced against G3 and G5 porcine strains (10), with G1, G2, and G4 serotype-specific MAbs (37), and with MAbs 159, 57/8, and YO-1E2, which have various degrees of cross-reactivity by neutralization and/or by ELISA with G3 strains (Fig. 2). Neutralization resistance closely corresponded with lack of binding to G11-specific MAbs, except for mutant A253-8D10/6E10, which was still bound by MAbs 5E6 and 8E8, although at a much lower avidity than the parental variant strain A253-8D10. None of the mutants reacted by ELISA with MAbs raised to G3 or G5 porcine strains or to G1, G2, and G4 serotype-specific MAbs (data not shown). MAbs 159, 57/8, and YO-1E2 were not reactive with either the parental G11 strains YM and A253 or with any of the variants (Fig. 2), with the exception of variant YM-5E6.1, which sustained a mutation at position 96 (Asp for Asn) (Fig. 3). At the same concentration of infectious virus particles,

Virus Strain (P, G type)	Amino Acid Sequence of Indicated Antigenic Hypervariable Regions and the Corresponding Amino Acid Positions			Monoclonal Antibody Reactivity Neutralization/ELISA								
	A (aa 87-101)	C (aa 208-224)	F (aa 235-242)	3C8	7F10	6E10	8E8	5E6	8D10	57/8	159	YO-1E2
A253 (P9[7], G11)	R E A A T Q I A D D K W K D T	L T T D P T T F E E V A S T E K L	H K L D V T T A	+/+	+/+	+/+	+/+	+/+	+/+	-/-	-/-	-/-
A253-3C8	I - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - -	- - - - - - - -	-/-	-/-	-/-	-/-	-/±	+/+	-/-	-/-	-/-
A253-3C8/8D10	I - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - -	- - - N - - - -	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
A253-6E10	T - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - -	- - - - - - - -	-/-	-/-	-/-	-/-	-/-	+/+	-/-	-/-	-/-
A253-8E8	T - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - -	- - - - - - - -	-/-	-/-	-/-	-/-	-/-	+/+	-/-	-/-	-/-
A253-8E8/8D10	T - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - -	- - - N - - - -	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
A253-5E6	T - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - -	- - - - - - - -	-/-	-/-	-/-	-/-	-/-	+/+	-/-	-/-	-/-
A253-7F10	G - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - -	- - - - - - - -	-/-	-/-	-/-	-/-	-/-	+/+	-/-	-/-	-/-
A253-8D10	- - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - E -	- - - - - - - -	+/+	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-
A253-8D10/6E10	- - - - I - - - - - - - - - -	- - - - - - - - - - - - - - - - E -	- - - - - - - -	-/-	-/-	-/-	-/±	-/±	-/-	-/-	-/-	-/-
A253-8D10/7F10	T - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - E -	- - - - - - - -	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
A253-8D10/8E8	G - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - E -	- - - - - - - -	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
YM (P9[7], G11)	H - - - - - - - - - - - - - -	- - - - - - - - - - - - - - A - - -	- - - - - - - -	-/-	-/-	-/-	+/+	+/+	+/+	-/-	-/-	-/-
YM-5E6.1	H - - - - - - - - N - - - - -	- - - - - - - - - - - - - - A - - -	- - - - - - - -				-/-	-/-	-/+	-/+	-/+	-/+
YM-5E6.5	H - - - A - - - - - - - - - -	- - - - - - - - - - - - - - A - - -	- - - - - - - -				-/-	-/-	-/+	-/-	-/-	-/-
YM-8E8	H - - - A - - - - - - E - - -	- - - - - - - - - - - - - - A - - -	- - - - - - - -				-/-	-/-	-/+	-/-	-/-	-/-
SA11 (P[2], G3)	T - - - - E - N - N S - - - -	- - - - A - - - - - - - - T A - - -	- - - S - - - -	-/-	-/-	-/-	-/-	-/-	-/+	+/+	+/+	+/+
RRV (P5B[3], G3)	T - - - - E - N - N S - - - -	- - - - T A - - - - - - - T A - - -	- - - S - - - -	-/-	-/-	-/-	-/-	-/-	-/-	+/+	+/+	+/+

FIG. 2. Relationship between amino acid substitutions in antigenic variants and reactivity with anti-VP7 MAbs in neutralization and ELISA. Dashes indicate identical amino acids. Neutralizing titer was determined by FFNA (7). Code used in neutralization: +, highly sensitive (reciprocal titer <8-fold compared to parental strain); ±, slightly sensitive (8 < reciprocal titer < 16-fold compared to parental strain); −, resistant (reciprocal titer > 16-fold compared to parental strain). Code used for ELISA: +, OD value >500; ±, OD value between 200 and 499; −, OD value < 200. Data with serotype-specific MAbs 3C8, 7F10, 6E10, 8E8, 5E6, and 8D10 were extracted from a previously published article (7), with the exception of the new data added with the double antigenic mutants and all reactions with cross-reactive MAb 57/8 (4, 7, 20, 28), MAb YO-1E2 (37), and MAb 159 (18, 36). Sequences of VP7 regions A, C, and F of YM (35); A253, A253-3C8, A253-6E10, A253-8E8, A253-5E6, A253-7F10, A253-8D10, YM-5E6.1, YM-5E6.5, and YM-8E8 (7); and SA11 and RRV (32) are from published reports.

ELISA optical density (OD) readings with this mutant were lower than with the RRV strain, probably due to the specificity of the capture antibody. Despite the high binding activity, none of the three MAbs showed any neutralizing activity (<1/100) with the variant YM-5E6.1 (Fig. 2), even after addition of an excess anti-mouse antibody to the neutralization mixture (data not shown).

Neutralization patterns of the immunoadsorbed anti-RRV rotavirus hyperimmune serum 32251. The neutralization reactivity of the serum, adsorbed separately with RRV, A253, and A253-5E6, decreased dramatically against each of the strains used for immunoadsorption (Table 3). Anti-RRV serum 32251 adsorbed with A253 was capable to reduce four times its neutralizing activity against RRV (G3), suggesting that some antibodies present in this particular serum recognize epitopes on the molecule of VP7 of porcine strain A253 (G11). Furthermore, RRV was at least eight times more resistant to neutralization with the serum adsorbed with the variant A253-5E6, whose mutation created a substitution which is found in most serotype G3 rotaviruses at position 87 (9, 21, 32).

DISCUSSION

During the study of antigenic variants of serotype G11 porcine strains, we came across a complex situation in which some antigenic variants, which had been selected with monotype- and serotype-specific MAbs (7), exhibited an increased recognition in neutralization assays by antisera raised against some serotype G3 strains and in binding by MAbs (MAbs 159, 57/8, and YO-1E2) that react with G3 strains by ELISA. This phenomenon was associated in all cases with a substitution in the antigenic variants of an amino acid identical or chemically similar to the amino acid present in the same position in serotype G3 strains in variable region A, one of the six variable regions on VP7 previously shown to be involved in virus neutralization (7, 10, 12, 14, 15, 20, 23, 24, 27, 28, 32, 38). This finding suggests that the positions involved, 87 and 96, correspond to key contact residues (conserved in all G3 strains except murine strains) of immunodominant epitopes which define serotype G3 rotavirus strains for the anti-serotype G3 sera used and for MAbs 159, 57/8, and YO-1E2, respectively.

The increased susceptibility of serotype G11 antigenic variants to G3 specific guinea pig antisera was observed with two different simian strain RRV (P5B[3]), a canine strain CU-1 (P5A[3]), and equine strains H-2 and FI14 (P11[12]) antisera. However, no reactivity was observed with rabbit or guinea pig sera raised against other serotype G3 strains: porcine A411, A131, and A138 (P9[7], the same P type as the mutants), human P (P1A[8]), canine K9 (P5A[3]), lapine ALA (P[14]), and murine EB (P10[16]) strains. Possible explanations are the following. (i) Slight alterations in the epitopes on VP7 antigenic regions A and C may influence the immunogenicity of the presented VP7 neutralization epitopes since most of the serotype G3 strains have an identical region A but all have a slightly different region C (7, 10, 12, 15, 23, 24, 27, 28, 38). Comparison of the VP7 antigenic region C sequences of the rotavirus strains RRV, H-2, FI14, and CU-1, whose antisera neutralized the antigenic variants, reveals a divergent group of amino acids (aa 212 and 213 and aa 220 and 221) within region C (Fig. 1) that might have influenced the reactivity of the sera. However, since no unique amino acid sequences in region C could be identified among these strains; analyses of more G3 serotype-specific hyperimmune sera are required. (ii) The interactions between VP7 serotype G3 with different VP4 types could affect the presentation of neutralization epitopes on VP7 and its immunogenicity (5). Since both K9 and CU-1 strains are P5A[3], these data suggest that the difference observed with the antisera raised against these two strains might be related to differences in the VP7 sequences, but the VP7 se-

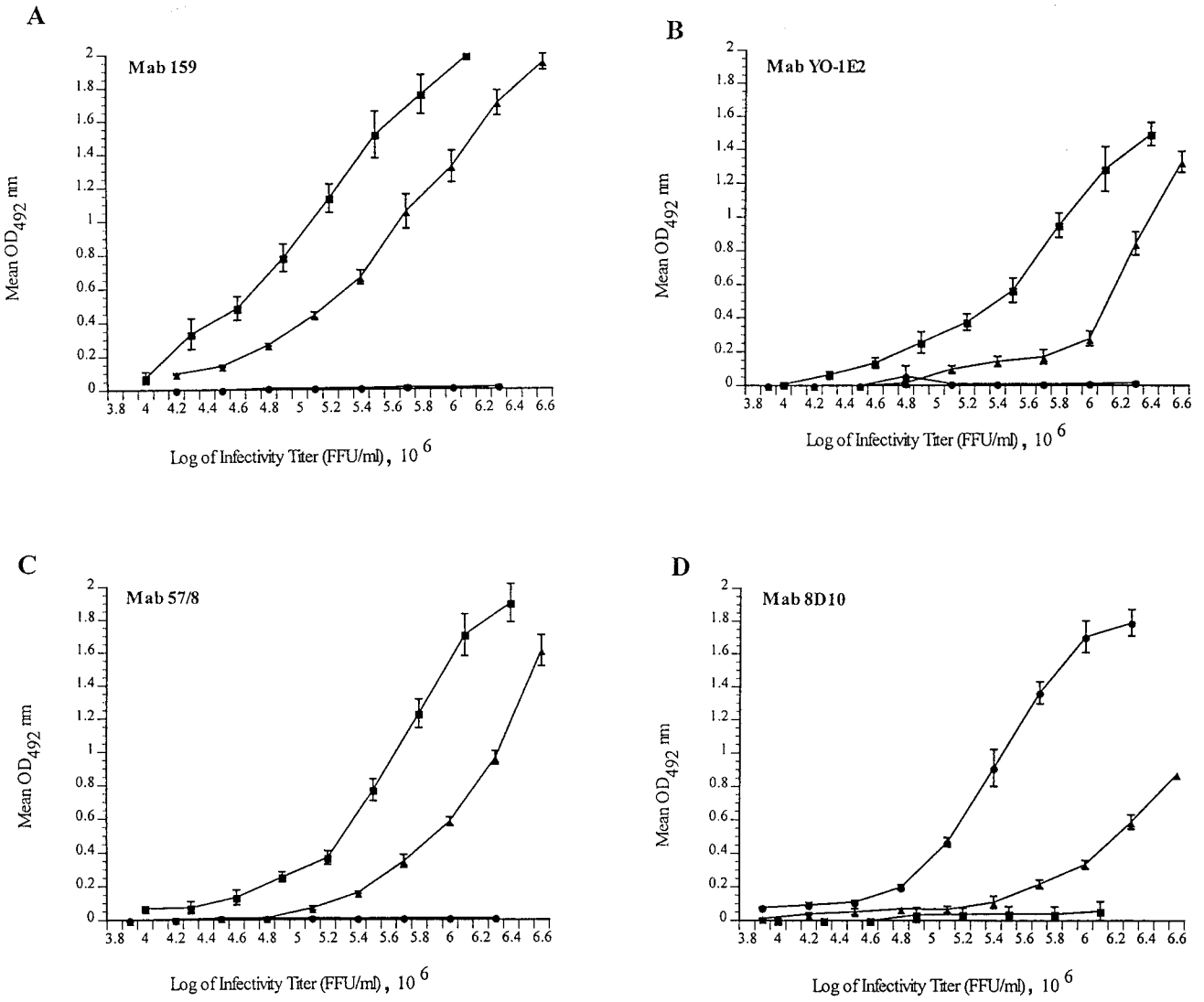

FIG. 3. Antibody binding curves, determined by ELISA, of MAbs 159, YO-1E2, 57/8, and 8D10 used as capture antibodies against serial dilutions of rotavirus strains RRV (G3) (■), and YM (G11) (●) and the variant YM-5E6.1 (G11/G3) (▲) and developed with a polyclonal serum to A253 (G11) (7). A similar concentration of infectious particles, measured by fluorescent focus assay (10^6 FFU/ml), was used as an approximate of the concentration of triple-layered particles. OD_{492}, OD at 492 nm.

quence of K9 is not known. (iii) Alternatively, there could be variation between species or individuals within a species in the immunodominance of epitopes (1). The differences observed with two anti-RRV guinea pig sera might be due to variation between individual animals within the same species and the host's immunological regulatory mechanisms, which may be important factors influencing the outcome of the immune response (1, 26).

TABLE 3. Neutralizing activity of the adsorbed anti-RRV hyperimmune serum

Virus	Reactivity[a]							
	Anti-RRV hyperimmune serum		Anti-RRV hyperimmune sera immunoadsorbed with virus[b]					
			−10 mM EDTA			+10 mM EDTA		
	−10 mM EDTA	+10 mM EDTA	RRV	A253	A253-5E6	RRV	A253	A253-5E6
RRV	1	2	>64	4	8–16	64	2–4	4–8
A253	32	32–64	>64	>64	>64	64	64	64
A253-5E6	4	8	>64	64	>64	64	32	64

[a] Expressed as the ratio of the reciprocal value of neutralization titer with anti-RRV hyperimmune serum 32251 (1/51,200) before preadsorption with indicated rotavirus strains and treatment with 10 mM EDTA as determined by FFNA (7).

[b] Immunoadsorption was carried out separately with rotavirus strains RRV, A253, and antigenic variant A253-5E6.

A second mutation selected with MAb 8D10 in the single mutants (A253-3C8 and A253-8E8) that exhibited dual serotype reactivity (G11/G3) did not allow anti-G3 sera to continue to neutralize the variants eliminating the dual serotype reactivity of the original mutants afforded by the presence of either Thr or Ile at position 87 on antigenic region A. MAb 8D10 selected a mutation at position 238, creating a new glycosylation site, that probably blocked access of neutralizing antibodies to region A present in all anti-G3 sera except anti-RRV serum 32251. Certainly, it has been reported that glycosylation at this site would prevent neutralization by blocking access to antigenic region A (20, 27). The fact that anti-RRV serum 32251 neutralized the double mutants (albeit to a lower extent) may be due to the nature of the antibodies present in this particular serum. Also, only the serotype G14 equine strain FI23 was neutralized by anti-RRV serum 32251 but not by anti-RRV serum 13610. Both P[12], G14 equine strains FI23 and FR4, isolated in North America and Venezuela, respectively, possess a Thr at position 87 in the antigenic region A of the VP7, but only FR4 has a potential glycosylation site at position 238 (region F) which may block access to epitopes in region A (3, 8, 22).

Cross-adsorption studies provided evidence that adsorption of RRV serum 32251 with one of the susceptible mutants (A253-5E6) decreased the neutralizing activity of the serum with RRV and vice versa, supporting the specificity of the effect. Interestingly, adsorption of the serum with the parental strain A253 decreased the reactivity of the serum with the escape mutant but not with RRV. Possible explanations are that antibodies which mediate neutralization of the variant bind, but do not neutralize, the parental strain and that neutralization of the variant strain requires, in addition to antibodies reactive with the epitope(s) mimicking the RRV one(s), antibodies to sites present in the parental strain A253.

Although it has been reported that conversion from one monotype to another may occur with a single amino acid change (13), the same has yet to be proven for G serotype specificity. Our novel results suggest that the serotype G11 variants that sustained amino acid substitutions at positions 87 and 96 of the same nature as or identical to the ones in all serotype G3 strains lifted constraints on the three-dimensional folding of the VP7 protein sufficiently to allow antigenic region A to become exposed to those anti-G3 hyperimmune sera whose antibodies were directed primarily to that site. However, a single amino acid substitution may not be enough to completely affect the serotype reactivity since all variants were neutralized efficiently by G11 sera. The question still remains as to how many substitutions are required so these mutants are no longer neutralized efficiently by serotype G11 antiserum. Rabbit antiserum directed against the antigenic variant A253-5E6 failed to neutralize any serotype G3 strain. Although every amino acid substitution can elicit antibodies, (i) the animal immunized with this mutant probably did not generate sufficient specific antibodies for the epitope involving Thr at position 87 of VP7 protein or (ii) Thr or Ile at position 87 may be immunodominant in the serotype G3 context but not in the serotype G11 context. Recently, it was reported that the antibody response in most hyperimmune sera raised to the hemagglutinin protein of type A influenza virus was, although polyclonal, biased to a single epitope (25).

The relatedness of VP7 genes of G11, G5, and G3 rotavirus strains is closer than that of other G serotypes (9, 35). It was interesting that certain G11 mutants behaved like G11/G3 viruses and not like G11/G5 viruses since amino acid relatedness between the VP7 protein of serotype G11 is closer to that of serotype G5 than to that of serotype G3 (9). It may be possible that the amino acid substitutions in the mutants correspond to key amino acids present in serotype G3 strains and not in G5 strains. Nevertheless, the G11 serotype may have arisen from either a G3 or G5 strain, which may have in turn continued to accumulate mutations due to the host selective immune pressure in a way similar as to that suggested by others (30, 31). Strains belonging to these different G serotypes (G3, G5, and G11) may cluster separately, but as mutations on VP7 accumulate, different interserotypic strains may start to approach another serotype. Therefore, the mechanism for antigenic drift may be unique to these three closely related rotavirus serotypes, and its relevance to rotavirus serotypes with greater sequence diversity in VP7 remains to be determined.

In any case, single point mutations might affect the serotype reactivity of G11 strains, originating virus with dual serotype specificity as measured with either monoclonal or polyclonal immunoreagents. A naturally occurring virus with a mixed G serotype reactivity (G3/G5) has been identified in pigs (30). However, why it has not been possible to detect more natural variants with dual serotype specificity is not known. Our results provide further evidence that "new" rotavirus serotypes may be introduced through the successive accumulation of mutations. It is likely that rotavirus strains could evolve into variants that no longer possess such a consensus sequence of amino acids that defines its serotype specificity (17), yielding virus progeny of another serotype specificity. Precise evaluation of the antigenic diversity is of critical importance for the development of effective vaccination strategies to prevent the appearance of "new" rotavirus serotypes.

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