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VP4 Monotype Specificities among Porcine Rotavirus Strains of the Same VP4 Serotype

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The porcine rotavirus OSU strain was used to produce monoclonal antibodies (MAbs) directed against the outer capsid protein VP4. From two separate fusions, eight MAbs that inhibited hemagglutination activity of the OSU strain were selected. All MAbs immunoprecipitated both the OSU VP4 protein derived from a lysate of infected MA104 cells and the OSU VP4 protein expressed in Sf9 cells by a recombinant baculovirus. By immunoprecipitation of in vitro-translated OSU gene 4 transcripts of different length, the eight MAbs were found to be specific for the VP8 subunit of VP4. All MAbs neutralized the OSU strain but failed to neutralize human, bovine, and simian rotavirus strains. Antiserum to the expressed OSU VP4 protein was used to study the distribution of VP4 antigenicity among porcine rotaviruses. At least two distinct specificities were identified among 14 rotavirus strains that had been previously assigned to four distinct VP7 serotypes. Five groups of monotype specificities of the VP4 protein were identified by the eight anti-VP4 MAbs among 11 porcine strains that share the same VP4 serotype.

Rotaviruses have emerged as a major etiological agent of gastroenteritis in humans and in several animal species. Two surface proteins of group A rotaviruses, VP7 and VP4, have been shown to independently elicit neutralizing antibodies and induce protective immunity (8, 9, 23). At least four VP7 serotypes have been described in porcine group A retroviruses. According to the classification scheme proposed by Hoshino et al. (10) and updated by Estes and Cohen (4), porcine rotavirus isolates have been classified as serotypes 3, 4, 5, and 11 and as having a mixed reactivity of serotypes 3 and 5 (1, 2, 18, 19, 25). Additional strains with unique antigenic characteristics have been described but not fully characterized (17, 24).

The outer capsid protein, VP4, has been associated with trypsin-enhanced infectivity (5) and with viral hemagglutination. The relevance of VP4 of porcine rotavirus strains in inducing a neutralizing response after oral infection has also been documented (8). However, little is known about VP4 antigenic polymorphism among rotavirus porcine strains. At least two alleles of the VP4 gene (OSU strain VP7 serotype 5 and Gottfried strain VP7 serotype 4) have been recognized on the basis of sequence comparisons and serological data (7, 21). On the basis of amino acid homology, the VP4 allele of the Gottfried strain has been shown to be related to the VP4 allele associated with human asymptomatic strains (7) and to share neutralizing epitopes with both virulent and asymptomatic human strains (11).

In the present report, a panel of monoclonal antibodies (MAbs) directed against the VP4 outer capsid protein of the porcine rotavirus OSU strain is described. These VP4 MAbs, which were found to be specific for the VP8 subunit of VP4, allowed the classification of five groups of monotype specificities in porcine rotavirus field isolates that share the same VP4 serotype.

Porcine rotavirus strains used included isolates from Argentina (provided by R. Bellinzoni and N. Mattion, Centro de Virologia Animal, Capital Federal, Argentina), Mexico (strain YM, supplied by C. Arias, Universidad Autonoma de Mexico, Cuernavaca, Mexico), the United States of America (USA) (strain SB-1A, supplied by L. Saif, Ohio State University; strains ISU-64 and ISU-65, supplied by P. Paul, Iowa State University, Ames), and Venezuela. The VP7 specificities of some strains have been previously described (1, 10, 17, 24, 25). Venezuelan strains A1.3, A253.1, A130.1, A46, and A34 were classified as the indicated serotypes (Table 1) on the basis of two-way neutralization assays against strains OSU, Gottfried, MMU18006, and YM and their respective hyperimmune sera (14).

The VP4 OSU MAbs were produced by using the porcine rotavirus OSU strain, grown in maintenance medium without trypsin (27). Purified preparations of the virus were used for immunization of BALB/c female mice. A standard immunization schedule and fusion protocol were used (13). Supernatants of hybridomas were tested by a sandwich enzyme-linked immunosorbent assay (13) and by inhibition of hemagglutination of goose erythrocytes, basically as previously described (12). From two separate fusions, 8 MAbs with hemagglutination inhibition activity were selected for further characterization. All MAbs immunoprecipitated a 85-kDa protein from a lysate of strain OSU-infected MA104 cells. The mobility of this protein was similar to that of VP4 immunoprecipitated by OSU rotavirus antiserum (Fig. 1). The specificity of these MAbs for the OSU VP4 protein was further confirmed by immunoprecipitation of the OSU VP4 protein from labeled lysates of *Spodoptera frugiperda* (Sf9) cells infected with a recombinant baculovirus (pAc373), which carries OSU gene 4 (20; data not shown).

To identify which of the two cleavage products of VP4 (VP5 or VP8 subunit) is recognized by the eight MAbs, the sequences that code for the VP8 subunit (amino acids 1 to 242) and for the polypeptide that represents amino acids 1 to 514 of the VP4 protein were transcribed in vitro from the cloned OSU gene 4 (21). The two transcripts were translated

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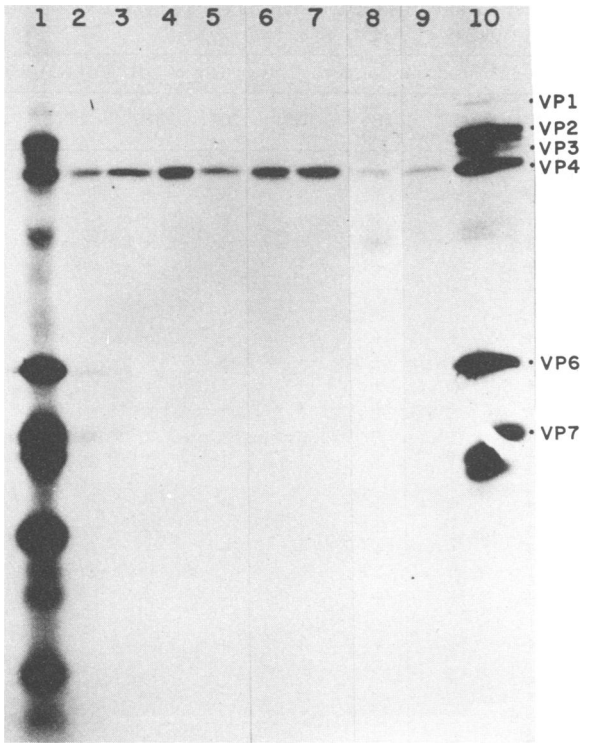

FIG. 1. Reactivity of MAbs with the VP4 protein of porcine rotavirus OSU strain. [^{35}S]methionine-labeled proteins of MA104 cells infected with the OSU strain were immunoprecipitated with OSU antiserum and ascitic fluid of the indicated hybridomas. Total [^{35}S]methionine protein lysates of OSU virus-infected cells (lane 1) immunoprecipitated with MAbs 3G5, 5D9, 1C11, 4B2, 2D5, 2C9, 2B6, and 3E7 (lanes 2 to 9, respectively) and with anti-OSU hyperimmune rabbit serum (lane 10) are shown.

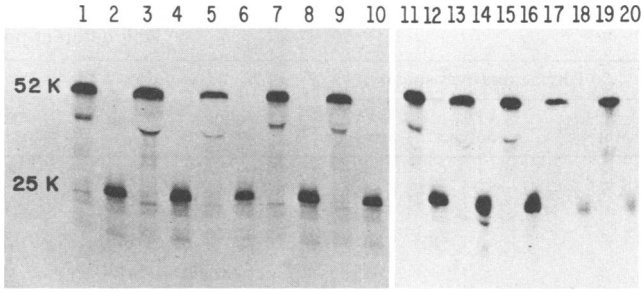

FIG. 2. Specificity of the VP4 OSU NMAbs to the VP8 subunit of VP4. The sequences for amino acids 1 to 242 (VP8 subunit) and for amino acids 1 to 514 of the VP4 of porcine OSU strain were amplified by the polymerase chain reaction, using as a template the cloned VP4 gene in the pTZ18R plasmid selected for the correct promoter orientation (21). Two primers were used to amplify the VP8 subunit of VP4, one that corresponds to the T7 promoter-conserved nucleotide sequence in pTZ18R and the second one complementary to nucleotides at positions 712 to 741 of the OSU gene 4 (5′-CACTATCTCTCTAGCCGATAAAGATACTGG-3′). To amplify the nucleotide sequence for amino acids 1 to 514, the T7 promoter primer was used together with oligonucleotide 5′-TCGC TATTTGCTGTGAC-3′, which is complementary to the nucleotide sequence at positions 1535 to 1551. The two amplified DNA fragments were transcribed by RNA polymerase T7, and the transcripts were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Each of the two translation products (representing amino acids 1 to 514 [odd-numbered lanes] and amino acids 1 to 242 [even-numbered lanes]) was immunoprecipitated with anti-OSU hyperimmune guinea pig serum (lanes 1, 2, 11, and 12), MAbs 2D5 (lanes 3 and 4), 2C9 (lanes 5 and 6), 3G5 (lanes 7 and 8), 2B6 (lanes 9 and 10), 3E7 (lanes 13 and 14), 4B2 (lanes 15 and 16), 1C11 (lanes 17 and 18), and 5D9 (lanes 19 and 20). Results from two different gels (lanes 1 to 10 and 11 to 20) are shown.

in a rabbit reticulocyte cell-free translation system in the presence of [^{35}S]methionine. The strategy and detailed conditions of the procedure have been described previously (6, 16). The eight MAbs immunoprecipitated both the small (25-kDa) and larger (52-kDa) polypeptides, indicating their specificity for the VP8 subunit of VP4 (Fig. 2).

Binding and neutralization activities of MAbs were quantified by immunofluorescence and a plaque reduction neutralization test, respectively, using the OSU strain. Immunofluorescence tests were performed in 96-well microtiter plates essentially as previously described (11), with the titer defined by the highest dilution giving a visible signal. Ascitic fluids of the OSU VP4 MAbs showed immunofluorescence titers varying between 1/1,280 and 1/5,120. By using a plaque reduction neutralization test performed basically as previously described (8), all MAbs were found to be able to neutralize the OSU strain at titers between 1/5,120 and 1/20,480 (data not shown). These OSU VP4 neutralizing MAbs (NMAbs) were found to be specific for VP4 epitopes not present in rotavirus strains of other species since they failed to neutralize human rotavirus strains KU, DS-1, and M37, bovine rotavirus strains NCDV and UK, and simian rotavirus strains SA11 and MMU18006 (data not shown). The human, bovine, and simian strains were chosen as representative of different alleles of the VP4 gene on the basis of sequence analysis (4) and on the basis of cross-neutralization studies with antisera against recombinant VP4 proteins for the human strains (6). The antigenic relationship between VP4 proteins of porcine strain OSU and 13 porcine strains belonging to at least four distinct VP7 serotypes was evaluated in a fluorescent-focus neutralization test (11), using guinea pig hyperimmune serum produced against baculovirus recombinant-expressed OSU VP4 (20) (Table 1). This hyperimmune serum neutralized 11 porcine strains tested at titers ranging from 1/640 to 1/2,560 but failed to neutralize strains A46, A34, and Gottfried (titers $<$ 1/80), the same strains which were not recognized by any of the anti-VP4 MAbs (Table 1).

Recently, antisera against recombinant VP4 proteins have been used to study VP4 antigenic polymorphism among human rotavirus strains (6). Three distinct serotypes and one subtype of the VP4 protein were identified in 17 human rotavirus strains that had been previously assigned to five distinct VP7 serotypes. The criterion for VP4 serotype specificity was the presence of an eightfold difference in neutralizing antibody titer (6). By this criterion, it appears that at least two VP4 serotypes are present among the 14 porcine strains that belong to at least four different VP7 serotypes. Eleven strains of porcine rotaviruses possessed a VP4 of the same serotype, designated porcine VP4 serotype 1. The Gottfried strain was classified as VP4 serotype 2. Further studies are required to establish the VP4 classification of strains A46 and A34, both with a VP7 serotype 5 specificity, which were not reactive with anti-OSU VP4 antiserum nor with any of the VP4 NMAbs.

Previous studies have shown that VP4 contains both heterotypic and homotypic neutralizing epitopes (15, 26). The amino-terminal VP8 subunit contains regions of greater genetic diversity and therefore is relatively serotype or strain

TABLE 1. Reactivity of OSU VP4 NMAbs and hyperimmune antiserum against baculovirus recombinant-expressed OSU VP4 protein with different porcine rotavirus isolates

Porcine rotavirus strains			Country of origin	Rotavirus strain	OSU VP4 antiserum[a]	Immunofluorescence reactivity pattern of OSU VP4 NMAbs[b]							
VP4 serotype	VP4 monotype	VP7 serotype				2D5	4B2	2B6	3G5	2C9	3E7	1C11	5D9
1	1	5	USA	OSU	2,560	+	+	+	+	+	+	+	+
		4	USA	SB-1A	640	+	+	+	+	+	+	+	+
		X[c]	USA	ISU-64	1,280	+	+	+	+	+	+	+	+
		X[c]	USA	ISU-65	640	+	+	+	+	+	+	+	+
		5	Venezuela	A130.1	2,560	+	+	+	+	+	+	+	+
		?	Venezuela	A138.1	2,560	+	+	+	+	+	+	+	+
	2	11	Mexico	YM	2,560	+	+	+	+	+	+	+	−
		3	Venezuela	A1.3	1,280	+	+	+	+	+	+	+	−
		11	Venezuela	A253.1	ND	+	+	+	+	+	+	+	−
		?	Venezuela	A8.2	ND	+	+	+	+	+	+	+	−
		?	Argentina	C135	ND	+	+	+	+	+	+	+	−
		?	Argentina	C60	ND	+	+	+	+	+	+	+	−
	3	?	Argentina	C91	640	+	+	+	+	+	+	−	−
		?	Argentina	C95.1	ND	+	+	+	+	+	+	−	−
		?	Argentina	C134	ND	+	+	+	+	+	+	−	−
	4	?	Argentina	C117	1,280	+	+	+	+	−	+	−	−
	5	?	Venezuela	A4.1	2,560	+	+	+	+	+	−	−	−
2		4	USA	Gottfried	<80	−	−	−	−	−	−	−	−
?		5	Venezuela	A46	<80	−	−	−	−	−	−	−	−
		5	Venezuela	A34	<80	−	−	−	−	−	−	−	−

[a] Fluorescent-foci neutralization titers of guinea pig hyperimmune serum against baculovirus-expressed OSU VP4 are expressed as the reciprocal of serum dilution inhibiting >66% of fluorescent foci. ND, not determined.
[b] Results are expressed as presence (+) or absence (−) of specific fluorescence.
[c] VP7 serotype different from serotype 4 or 5 (24).

specific, while the carboxy-terminal VP5 subunit contains primarily conserved amino acid regions (15, 26).

Since the VP4 OSU NMAbs recognized epitopes of the VP8 subunit of VP4, the antigenic variation of this subunit among 20 porcine rotavirus strains of different origin was tested by immunofluorescence of infected cells (Table 1). Consistent with the high degree of genetic diversity of VP8, reactivity of anti-VP8 MAbs was specific for different subsets of the porcine strains tested, indicating that antigenic variation occurred among strains that appeared to share the same type of VP4, as demonstrated by their reactivity with the anti-OSU recombinant VP4 serum. It was thus possible to distinguish five distinct groups of monotype specificities among the 11 porcine rotavirus isolates that share VP4 serotype 1 specificity. Six porcine rotavirus strains reacted with all VP4 NMAbs tested and were designated monotype group 1. Six strains were recognized by all the VP4 NMabs with the exception of NMAb 5D9 and thus formed a second group. Three strains (monotype group 3) were recognized by six of the eight NMAbs. Monotype group 4 represented by the porcine rotavirus C117 strain, reacted with five NMAbs and failed to be recognized by NMAbs 2C9, 1C11, and 5D9. Finally, porcine rotavirus A4.1 strain of monotype group 5 was distinguished from monotype group 4 on the basis of reactivity of NMAbs 2C9 and 3E7 (Table 1). Recently it has been determined that the VP8 subunit contains the major antigenic site(s) responsible for the VP4 serotype specificity of rotaviruses (6). Since the topographical relationship among the epitopes recognized by VP4 OSU NMAbs was not determined and the relative contribution of these epitopes to the definition of a VP4 serotype is not known, the significance of the variation in reactivity observed among the porcine strains with NMAbs cannot be determined at the present time. However, this variation might be analogous to that observed among individual epitopes related to a VP7 serotype specificity. Rotavirus strains of the same VP7 serotype that have one amino acid substitution in a particular epitope and have therefore lost the ability to be neutralized by a MAb have been classified as monotypes (3, 22).

In the present report, the use of the term monotype has been expanded to include antigenic variants within a serotype detected by a binding-type assay rather than by a neutralization assay. Sequence analysis of escape mutants of porcine rotavirus OSU strain produced with the OSU VP4 NMAbs and comparison of the nucleotide sequences of the VP4 gene of porcine rotavirus isolates may help to localize the epitopes responsible for VP4 monotypes.

The finding that 11 porcine rotavirus strains belonging to at least four VP7 serotypes were neutralized by antiserum to the VP4 of OSU indicates that the latter strain may induce protective immunity against a broad range of strains belonging to this VP4 serotype. However, the relevance of monotype variation in the natural history of porcine rotavirus infection must be elucidated.

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