Molecular and Antigenic Characterization of Porcine Rotavirus YM, a Possible New Rotavirus Serotype

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In 1983, we isolated a porcine rotavirus (strain YM) that was prevalent in several regions of Mexico, as judged by the frequency of its characteristic electrophoretotype. By a focus reduction neutralization test, rotavirus YM was clearly distinguished from prototype rotavirus strains belonging to serotypes 1 (Wa), 2 (S2), 3 (SA11), 4 (ST3), 5 (OSU), and 6 (NCDV). Minor, one-way cross-neutralization (1 to 5%) was observed when antisera to the various rotavirus strains were incubated with rotavirus YM. In addition, the YM virus was not neutralized by neutralizing monoclonal antibodies with specificity to serotypes 1, 2, 3, and 5. The subgroup of the virus was determined to be 1 by enzyme-linked immunosorbent assay. To characterize the serotype-specific glycoprotein of the virus at the molecular level, we cloned and sequenced the gene coding for VP7. Comparison of the deduced amino acid sequence with reported homologous sequences from human and animal rotavirus strains belonging to six different serotypes further supported the distinct immunological identity of the YM VP7 protein.

Rotaviruses are the most common etiologic agents of acute gastroenteritis in children under 2 years of age (28, 31). They are also associated with diarrhea in the young of many animal species (19). These viruses were first reported in association with human infantile gastroenteritis in 1973 (6). Since then, knowledge of rotaviruses has accumulated very rapidly because of their great medical and veterinary importance.

The virions are composed of an RNA genome and a double-layered capsid (31). The genome is made up of 11 segments of double-stranded RNA (dsRNA), with sizes ranging from about 650 to 3,500 base pairs (8, 41). The capsid consists of at least five protein classes, three of which (VP1, VP2, and VP6) make up the inner layer, whereas the other two (VP3 and VP7) are in the surface layer of the virus (17, 18). Both VP3 and VP7 are able to elicit antibodies capable of neutralizing virus infectivity (1, 5, 26, 36, 39, 46). VP7, a 37,000-molecular-weight protein, is glycosylated and exposed on the outer capsid of the virion (4, 16). Studies with reassortant viruses have shown that the serotype-specific neutralizing phenotype segregated with the gene that encodes this protein (gene segment 7, 8, or 9, depending on the rotavirus strain) (21, 30, 47). Serotypes of rotavirus are defined on the basis of the neutralization of viral infectivity, and, to date, four serotypes (serotypes 1 to 4) of rotavirus have been identified among strains recovered from humans (29). Recently, two candidates for new human rotavirus serotypes have been proposed (12, 35). In animals, at least five serotypes have been identified (serotypes 3 to 7), but, by neutralization assays, two of the animal serotypes (serotypes 3 and 4) have been shown to cross-react with the corresponding human rotavirus serotypes (29). All rotavirus strains isolated from pigs have been classified as either serotype 4 or 5, with the Gottfried and OSU strains representing the prototype virus, respectively (7, 29). Recently, a porcine rotavirus antigenically related to human rotavirus serotype 3 has been found in Australia (38).

In this article we report the characterization of a rotavirus which was isolated from a diarrheic piglet in Sonora, Mexico, and which, by immunological and molecular criteria, seems to belong to a different serotype from those previously reported.

MATERIALS AND METHODS

Virus isolation. The porcine rotavirus (strain YM) was isolated from a diarrheic piglet in Sonora, Mexico. The intestinal content was suspended (10%, vol/vol) in Eagle minimal essential medium containing 250 μg of trypsin (1:250; Difco Laboratories) per ml. The suspension was clarified by centrifugation at 2,000 × g for 30 min. The supernatant fluid (0.2 ml) was inoculated into thrice-washed tubes of confluent MA104 cells. After an adsorption period of 1 h at 37°C, cultures were fed with Eagle minimal essential medium and incubated in a roller apparatus at 37°C. Serial passages were performed with suspensions of the whole culture treated with 10 μg of trypsin per ml for 1 h at 37°C. In passage 5, a complete cytopathic effect was observed at 16 h postinfection.

Virus and cells. Rotaviruses Wa, S2, and ST3 were obtained from Y. Hoshino, National Institutes of Health, Bethesda, Md.; NCDV was obtained from R. S. Spendlove, Utah State University, Salt Lake City, Utah; SA11 was obtained from H. H. Malherbe, Gull Laboratories, Salt Lake City, Utah; and OSU was obtained from L. Saif, Ohio State University, Wooster, Ohio. All rotavirus strains were grown in MA104 cells and purified as previously described (17).

Immune sera. Hyperimmune sera against rotaviruses YM and OSU were prepared in mice essentially as described by Arias et al. (2), except that the animals received only three injections (50 μg each) of the corresponding purified virus. Hyperimmune sera to Wa, S2, ST3, SA11, and NCDV were prepared in rabbits as described (4). The neutralizing monoclonal antibodies 5E8 (serotype 1), 1C10 (serotype 2), 4F8

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(serotype 3), and 5B8 (serotype 5) and the subgroup-specific monoclonal antibodies 255/60 (subgroup I) and 631 (subgroup II) were kindly provided by H. B. Greenberg, Stanford University, Stanford, Calif. (25, 42, 43).

**Subgroup and serotype characterization of YM.** The subgroup of rota virus YM was determined by an enzyme-linked immunosorbent assay with monoclonal antibodies (25). The neutralization antigen serotype of rota virus YM was determined by an immunoperoxidase focus reduction neutralization assay as described by Arias et al. (2).

**Molecular cloning and nucleic acid sequencing.** The 11 dsRNA segments derived from rota virus YM were cloned as described by López et al. (manuscript in preparation). The recombinant clones containing hybrid plasmids with cDNA inserts corresponding to YM RNA segment 9 were selected by hybridization of random recombinant plasmids, labeled by nick translation, to YM RNA segment 9 isolated by gel electrophoresis (4). The inserts in the selected clones were sequenced by the procedure of Maxam and Gilbert (37). To obtain the sequence of the 5' and 3' ends of the gene, which were not present in the selected clones, the isolated dsRNA segment 9 was hybridized with synthetic oligonucleotides complementary to regions 127 to 141 and 978 to 993 of the plus and minus strands of RNA segment 9, respectively, and the sequence was determined by the dideoxy-chain termination method with avian myeloblastosis virus reverse transcriptase as described (33).

**Nucleic acid hybridization.** SA11 and YM viral RNAs were electrophoresed in a 5% acrylamide gel (acylamide/bisacrylamide ratio, 20:0.5) in Tris-borate buffer (0.1 M Trizma base [Sigma Chemical Co.] and 0.002 M EDTA, adjusted to pH 8.3 with boric acid). Viral RNAs were transferred to nitrocellulose paper and hybridized to nick-translated plasmid pSR965, which contained a full-length cDNA copy of SA11 gene 9 (3), essentially as described by López et al. (32).

**RESULTS**

**Isolation.** For virus isolation, an intestinal-content suspension from a diarrheic piglet was treated with trypsin and inoculated into roller-tube cultures of MA104 cells. After 4 days of incubation of passage 1, approximately 50% of the cells showed a cytopathic effect. By passage 5, when the virus was inoculated at high multiplicity of infection, a complete cytopathic effect was observed at 16 h postinfection.

After eight passages in MA104 cells, the virus was still able to cause profuse diarrhea in colostrum-deprived 1-day-old pigs.

**dsRNA electrophoretic profile.** The electrophoretic pattern of the YM rotavirus dsRNA could be readily distinguished from the corresponding patterns of the human rotavirus strains Wa, S2, and ST3, the simian rotavirus SA11, the porcine rotavirus OSU, and the bovine rotavirus NCDV (Fig. 1). The electrophoretotype of the cell culture-adapted YM strain was identical to that obtained from the original sample from which the virus was isolated (data not shown).

In 1983, the year rotavirus YM was isolated, its characteristic electropherotype was observed in 62 of 63 rotavirus-positive samples collected from five of the most important porciculture areas of Mexico, located in the states of Jalisco, Veracruz, Sonora, Michoacan, and Edo. de Mexico (A. M. Ruiz and A. Morilla, unpublished results).

**Subgroup determination.** The subgroup of the YM rotavirus strain was determined to be I by the enzyme-linked immunosorbent assay with subgroup-specific monoclonal antibodies (25). This subgroup specificity is the same as that reported for the OSU strain but different from Gottfried, the other reference porcine rotavirus strain, which has been shown to belong to subgroup II (29).

**Serotype determination.** The YM rotavirus strain was determined, by the focus reduction neutralization test (2), to be antigenically distinct from rotavirus serotypes 1 to 6, on the basis of >20-fold differences between titers of homologous and heterologous reciprocal neutralizing antibodies. Hyperimmune mouse antisera to rotavirus YM did not significantly neutralize prototype rotavirus strains of serotypes 1 (Wa), 2 (S2), 3 (SA11), 4 (ST3), 5 (OSU), and 6 (NCDV) (Table 1). Rotavirus YM could also be assigned to a different serotype when antisera to these various rotavirus strains were tested against it. However,

![FIG. 1. dsRNA electrophoretic patterns of porcine rotavirus YM and rotavirus strains of different serotypes. Human rotavirus Wa (serotype 1), human rotavirus S2 (serotype 2), simian rotavirus SA11 (serotype 3), human rotavirus ST3 (serotype 4), porcine rotavirus YM (possible new serotype), porcine rotavirus OSU (serotype 5), and bovine rotavirus NCDV (serotype 6) are shown.](http://jvi.asm.org/)

**TABLE 1. Antigenic characterization of porcine rotavirus strain YM by the focus reduction neutralization test**

<table>
<thead>
<tr>
<th>Rotavirus (serotype)</th>
<th>Reciprocal of 60% FRN antibody titer of hyperimmune antiserum to indicated rotavirus (serotype)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wa (1)</td>
</tr>
<tr>
<td>Wa (1)</td>
<td>8,000 ^b</td>
</tr>
<tr>
<td>S2 (2)</td>
<td>16,000</td>
</tr>
<tr>
<td>SA11 (3)</td>
<td>--</td>
</tr>
<tr>
<td>YM</td>
<td>100</td>
</tr>
<tr>
<td>ST3 (4)</td>
<td>--</td>
</tr>
<tr>
<td>OSU (5)</td>
<td>--</td>
</tr>
<tr>
<td>NCDV (6)</td>
<td>--</td>
</tr>
</tbody>
</table>

* Homologous values are in boldface type. FRN, Focus reduction neutralization.

^b --, Not tested.
low-level cross-neutralization (1 to 5%) was observed with all these reference strains.

Further evidence that rotavirus YM does not belong to the previously established serotypes 1, 2, and 3 is that neutralizing monoclonal antibodies to these serotypes failed to significantly neutralize it. The homologous-to-heterologous reciprocal titer ratios for neutralizing monoclonal antibodies to serotypes 1, 2, and 3 were >160-fold, >640-fold, and >1,600-fold, respectively.

Cloning and sequencing of the serotype-specific glycoprotein gene. To characterize the serotype-specific glycoprotein at the molecular level, we cloned the rotavirus YM genome and sequenced gene 9, which was shown by Northern (RNA) blot hybridization to be homologous to the VP7 glycoprotein gene of simian rotavirus SA11 (data not shown). The general features of the YM glycoprotein gene (Fig. 2) are the same as those reported for homologous genes from other rotavirus strains. It was found to be 1,062 base pairs long, with an open reading frame of 978 bases, which started at nucleotide 49 with the first ATG and ended at nucleotide 1,025 with a TAG termination codon. The 5' and 3' untranslated sequences were 48 and 36 nucleotides long, respectively.

The open reading frame codes for a protein of 326 amino acids and contains a second in-phase initiation codon 30 triplets downstream from the first one. This second ATG has been shown to be functional as a start site in rotavirus SA11 (10, 44). The amino-terminal portion of the encoded protein contains two highly hydrophobic regions, one after each of the two in-phase potential initiation codons, and a carbohydrate acceptor site (Asn-Ser-Thr) at residue 69. This potential glycosylation site is conserved among all rotavirus strains but one (20). All the mentioned features, together with the eight cysteine residues present in the YM VP7 protein, are absolutely conserved among all reported rotavirus strains (3, 9, 13, 15, 20, 22, 23, 27, 34, 40; E. R. Mackow, R. D. Shaw, S. M. Matsu, P. T. Vo, D. Benfield, and H. B. Greenberg, Virology, in press).

Amino acid homology between the YM VP7 protein and the homologous proteins from animal and human rotavirus strains. When we compared the deduced YM VP7 amino acid sequence with VP7 sequences derived from other human and animal rotavirus strains, we found the following overall homologies: 76% with serotype 4 strains (ST3 and VA70); 77% (DS1, HN126, and Hu5) and 78% (S2) with serotype 2 strains; 77% (M37) and 78% (D, Mo, and Wa) with serotype 1 strains; 81% (UK) and 82% (NCDV) with serotype 6 strains; 87% with a serotype 5 strain (OSU); and
FIG. 3. Comparison of the YM amino acid sequence of VP7 regions A, B, and C with the corresponding regions of rotavirus strains belonging to six different serotypes. The amino acid sequences from Wa (40), HU5 (13), S2 (27), RRV (23; Mackow et al., in press), SA11 (3, 9), OSU (22), NCDV (20), UK (15), and D. Mo, M37, DS1, HN126, P, ST3, and VA70 (23) were compared with the amino acid sequence of YM. Dashes indicate identical amino acids. The serotype of each rotavirus strain is shown in parentheses.

86, 88, and 89% with strains P, SA11, and RRV, respectively, all of which belong to serotype 3.

The nucleotide sequence of rotavirus variants selected for their resistance to neutralization by VP7-directed, serotype-specific monoclonal antibodies has allowed the mapping of the sites involved in VP7-specific neutralization to three discrete regions in the protein (14, 32, 45). These three regions, defined as A, B, and C by Dyall-Smith et al. (14), have a high degree of divergence among different rotavirus strains but are highly conserved among rotavirus belonging to the same serotype (23). The comparison of the amino acid sequence of these selected regions of VP7 between YM and other rotaviruses (Fig. 3) showed that the YM VP7 sequence does not match any of the sequences derived from viruses belonging to six different serotypes, suggesting a novel VP7 specific neutralization identity of the porcine rotavirus YM.

DISCUSSION

A unified system for serotypic classification of human and other mammalian and avian strains of rotavirus has been proposed by Hoshino et al. (29). In their report, seven different serotypes were identified among the strains studied. Serotypes 1 and 2 were found only in human rotaviruses, serotypes 3 and 4 were found in rotavirus strains isolated from humans as well as from animal species, and serotypes 5 to 7 were exclusive to animal rotaviruses. Two recently reported rotavirus serotypes isolated from humans, which do not cross-react with the seven known serotypes (12, 35), have not been classified yet. In the work described in this report we have established the serotypic individuality of a porcine rotavirus isolate by cross-neutralization studies with prototype rotavirus strains belonging to serotypes 1 to 6. Since serotype 7 rotavirus strains have been isolated only from avian species (29), it is suggested that porcine rotavirus YM could represent a new rotavirus serotype.

The cross-neutralization studies clearly showed that hyperimmune mouse antisera to rotavirus YM did not neutralize rotavirus strains of serotypes 1 to 6, whereas minor cross-neutralization of YM by reference antisera to heterotypic rotaviruses was observed. This minor, one-way neutralizing cross-reactivity was <5%, the accepted limit for defining a distinct serotype (29).

It has been shown that neutralization in vitro and in vivo can be mediated by the two rotavirus surface polypeptides VP3 and VP7 (5, 26, 36, 39, 46). Comparison of the amino acid sequence of immunologically relevant regions of YM VP7 with the corresponding VP7 sequences of heterotypic rotavirus strains indicates that YM VP7 is unique, suggesting that VP3 might be responsible for the minor cross-neutralization observed. This observation is supported by the fact that VP7-specific neutralizing monoclonal antibodies to rotavirus serotypes 1, 2, 3, and 5 did not significantly neutralize rotavirus YM. In this regard, it is interesting that the one-way cross-neutralization of human rotavirus W161 by antisera to rotavirus strains of serotypes 1 to 4 was localized to VP3 by immunological analysis of reassortant viruses (12).

Rotavirus YM was assigned to subgroup I. This was not unexpected, since most animal rotavirus strains have this subgroup specificity (29). This observation clearly differentiates rotavirus YM from the porcine rotavirus Gottfried, which belongs to subgroup II, serotype 4 (29), and which was not available for this study.

In the last few years, the nucleotide sequence of the VP7-encoding genes from 17 human and animal rotavirus strains belonging to six different serotypes has been determined (3, 9, 11, 13, 15, 20, 22, 23, 27, 34, 40; Mackow et al., in press). Comparison of the deduced VP7 amino acid sequences of all those strains showed a high (91 to 100%) overall homology among strains of the same serotype, whereas the highest homology between two strains of different serotypes was 86% (23). When the rotavirus YM VP7 amino acid sequence was compared with those reported, it was found to be more closely related to serotype 3 strains (86 to 89% homology). However, this observation was not surprising, since pairwise comparisons between different serotypes showed that serotype 3 viruses shared the highest homology with the five other serotypes compared (23). YM VP7 had also a high identity (87%) with the VP7 of the porcine rotavirus OSU. This might reflect the common host origin of these two viruses. Escape mutations to neutralization by VP7-specific neutralizing monoclonal antibodies have been mapped to three different regions in the VP7 protein (14, 45; Mackow et al., in press). These three regions have a high degree of divergence among different rotavirus strains but are highly conserved among rotaviruses belonging to the same serotype (Fig. 3). When the sequences from two rotaviruses of the same serotype are compared, only one or two amino acid changes are found (23, 24). The amino acid sequences in these three regions of YM VP7 showed a fair number of differences from the other VP7 sequences, supporting the idea of a separate serotypic identity for YM VP7. However, it is interesting that region C of YM and serotype 3 rotaviruses showed some conservation, with only two amino acid changes (residue positions 212 and 220) between the SA11 and YM sequences. Since these two changes are not in the positions where escape mutations have been mapped (14, 45; Mackow et al., in press) and since an amino acid change at position 212 is also observed between strains ST3 and VA70 (23), both of which belong to serotype 4, it would be interesting to see whether monoclonal antibodies directed to the C region of serotype 3 viruses have some neutralizing activity on the YM infectivity.
It has been reported that three other regions of VP7 (amino acids 39 to 50, 120 to 130, and 233 to 242) are highly divergent between different rotavirus strains and very conserved among rotaviruses of the same serotype (23), thus being potentially serotype specific. However, in the work described in this report, two of those regions (amino acids 120 to 130 and 233 to 242) have been found to be identical between rotaviruses YM and RRV, with the last region also being identical with that in SA11. In addition, there was only one amino acid change in amino acids 120 to 130 between the YM and the SA11 and P sequences, and there was one change in amino acids 233 to 243 between the YM and the OSU, NCDV, and UK sequences. These observations, together with the facts that escape mutations have not been mapped to these regions (14, 45; Mackow et al., in press), suggest that they do not have a role (at least a major one) in the induction of neutralizing antibodies.

Recently, Benfield et al. (D. A. Benfield, E. A. Nelson, and Y. Hoshino. Abstr. VII Int. Congr. Virol., R11.19, p. 111, 1987) obtained a monoclonal antibody (57-8) which efficiently neutralized viruses of serotypes 3, 4, and 6. Analysis of the amino acid sequences of the serotype-relevant regions of VP7 among different rotavirus strains (Mackow et al., in press) showed that amino acids 99 to 101, 214, and 218 were conserved among viruses of these three serotypes and were different among viruses of serotypes 1, 2, and 5, which were not neutralized by monoclonal antibody 57-8. Interestingly, rotavirus YM shares the amino acid specificity in these positions with the viruses of serotypes 3, 4, and 6, making it a good natural probe with which to analyze the specificity and amino acid sequence requirements of monoclonal antibody 57-8 for rotavirus neutralization.

On the basis of the RNA genome electrophoretic pattern, rotavirus YM was widespread in Mexico in 1983, the year it was isolated; its characteristic electropherotype was observed in all but one of the porcine rotavirus-positive samples detected. However, in subsequent years, a systematic screening for rotaviruses in swine was not continued, and therefore we do not know whether the high prevalence observed for this electropherotype in 1983 was maintained over the following years or whether it was replaced by a different electropherotype. A serological survey of porcine samples for antibodies against OSU, Gottfried, and YM would help to reveal the incidence of infection of Mexican swine by these three viruses.

It seems clear, from the appearance of new rotavirus serotypes and from the various and complex patterns of cross-reactivity that have been observed, that the immunological relationships among different rotavirus strains are not as simple as initially thought. Thus, before safe and effective animal and human rotavirus vaccines can be designed, further structural and immunological studies, together with the determination of the relative prevalence of the different rotavirus strains, are needed.

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