Infection Immunity of Piglets to Either VP3 or VP7 Outer Capsid Protein Confers Resistance to Challenge with a Virulent Rotavirus Bearing the Corresponding Antigen

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A single-gene substitution reassortant 11-1 was generated from two porcine rotaviruses, OSU (serotype 5) and Gottfried (serotype 4). This reassortant derived 10 genes, including gene 4 encoding VP3, from the OSU strain and only gene 9, encoding a major neutralization glycoprotein (VP7), from the Gottfried strain and was thus designated VP3:5; VP7:4. Oral administration of this reassortant to colostrum-deprived gnotobiotic newborn pigs induced a high level of neutralizing antibodies not only to Gottfried VP7 but also to OSU VP3, thus demonstrating that VP3 is as potent an immunogen as VP7 in inducing neutralizing antibodies during experimental oral infection. Gnotobiotic piglets infected previously with the reassortant were completely resistant to oral challenge with the virulent Gottfried strain (VP3:4; VP7:4), as indicated by failure of symptoms to develop and lack of virus shedding. Similarly, prior infection with the reassortant induced almost complete protection against diarrhea and significant restriction of virus replication after oral challenge with the virulent OSU strain (VP3:5; VP7:5). Thus, it appears that (i) the immune system of the piglet responds equally well to two rotavirus outer capsid proteins, VP3 and VP7, during primary enteric rotavirus infection; (ii) antibody to VP3 and antibody to VP7 are each associated with resistance to diarrhea; and (iii) infection with a reassortant rotavirus bearing VP3 and VP7 neutralization antigens derived from two viruses of different serotype induces immunity to both parental viruses. The relevance of these findings to the development of effective reassortant rotavirus vaccines is discussed.

Diarrhea is a complex disease of the gastrointestinal tract and is of major importance in human and veterinary medicine; rotavirus is a major etiologic agent of this syndrome throughout the world (4, 5, 10, 16, 27). Several different approaches to immunoprophylaxis are now being taken with the aim of protecting infants and young children against severe rotavirus diarrhea, and these include the development and evaluation of (i) attenuated human rotaviruses, including cold-adapted rotavirus mutants; (ii) rotaviruses of animal origin which are restricted in humans; and (iii) intra- and interspecies reassortant rotaviruses (3, 9, 17-19, 21, 22, 28).

Rotaviruses have two major surface proteins, VP3 (gene 4 product) and VP7 (gene 8 or 9 product), which have been shown to be independent neutralization antigens (12, 24). Recent studies have shown that VP3 is as potent in inducing neutralizing antibodies as VP7 under conditions of parenteral or oral hyperimmunization (11, 12, 24, 25). More recently, mouse dams orally hyperimmunized with a reassortant rotavirus containing the VP3 of one serotype and the VP7 of another serotype were shown to confer passive protection to their progeny against challenge with either parental rotavirus (25).

In swine, the placenta is a barrier to the transport of maternal antibodies to the fetus. In this study, by using the homologous system of colostrum-deprived, newborn gnotobiotic pigs and a single VP7 gene substitution reassortant porcine rotavirus, we have studied (i) the immunogenicity of VP3 and VP7 under conditions of single experimental oral infection and (ii) the protective role of antibodies to VP3 and VP7 against challenge with virulent parental porcine rotaviruses. The observations made during this study have implications for the development of effective reassortant rotavirus vaccines.

MATERIALS AND METHODS

Viruses, cells, hyperimmune antisa, and PRN assay. The following viruses were used in this study: human rotavirus DS-1 (serotype 2), porcine rotavirus Gottfried (serotype 4), porcine rotavirus SB-1A (serotypes 4 and 5), and porcine rotavirus OSU (serotype 5). All rotaviruses were plaque purified three times in this laboratory before use in this experiment. The OSU and Gottfried strains used in the cross-protection studies were gut-origin viruses which had been serially passaged only in gnotobiotic piglets. An established cell line of fetal rhesus monkey kidney cells, MA104 (14), was used for virus propagation, production and selection of reassortant viruses, and the plaque reduction neutralization (PRN) assay. Hyperimmune antiserum against each of the viruses was prepared in guinea pigs. Hyperimmune antiserum was heat inactivated at 56°C for 30 min before it was used in the neutralization assay. The PRN assay was performed in plastic six-well plates (Costar, Cambridge, Mass.) of MA104 cell monolayers to measure neutralizing antibody, as previously described (15, 29).

Genetic reassortment, selection of reassortants, and geno-
type analysis of reassortant rotavirus. Genetic reassortment was carried out as previously described (11).

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RNA-RNA hybridization assays were performed to determine the parental origin of the genes present in reassortant rotaviruses, as described previously (6). Generation and genotype analysis of a single VP3 gene substitution OSU × DS-1 reassortant 46-1, which derived only its VP3 gene segment from its porcine OSU parent and 10 gene segments from its human DS-1 parent, was described previously (11).

**Experimental animals and cross-protection studies.** Gnotobiotic piglets used in this study were obtained, housed, and maintained as previously described (1). Individuals from two litters (20 piglets) of colostrum-deprived gnotobiotic piglets were treated orally with 2 ml each of one of the following preparations: Gottfried virus as a 50% suspension of intestinal contents from a gnotobiotic piglet which had a titer of 2 × 10^6 fluorescent-focus units (group 1), OSU virus as a 4% suspension of intestinal contents from a gnotobiotic piglet which had a titer of 2.5 × 10^6 fluorescent-focus units (group 2), or OSU × Gottfried reassortant 11-1 as a 1:2 dilution of infected MA104 cell lysate containing 5 × 10^5 PFU (groups 3 and 4). Two groups (groups 5 and 6) of piglets were designated controls and were not administered virus at age 4 to 5 days. All piglets, including the controls, were challenged orally 3 weeks later with 2 ml of either the OSU or the Gottfried virus preparation described above. Piglets were examined daily for evidence of diarrhea, and rectal swabs were collected daily for 2 weeks postchallenge. Virus shedding was examined by a cell culture immunofluorescence test (1) and in some cases by virus isolation in MA104 cell cultures. Blood samples were obtained at the time of primary infection, 3 weeks later at the time of challenge, and 2 weeks after challenge.

**RESULTS**

**Generation and characterization of a single-gene substitution OSU × Gottfried reassortant 11-1.** After mixed infection of MA104 cells with the porcine OSU and Gottfried rotaviruses, we isolated a single-gene substitution reassortant, 11-1, which derived only its VP7 gene segment from the Gottfried parent, whereas the remaining 10 gene segments were derived from the OSU parent (Fig. 1). Reassortant 11-1 was neutralized by hyperimmune antiserum raised against either OSU or Gottfried (Table 1). Also, hyperimmune antiserum to this reassortant neutralized the Gottfried and OSU strains to the same degree. It is of interest that a naturally occurring intertypic porcine rotavirus, SB-1A, with a VP3 of serotype 5 and a VP7 of serotype 4 was recently identified (Table 1) (11).

**Resistance in gnotobiotic piglets to both parental viruses induced by OSU × Gottfried reassortant rotavirus 11-1.** Previous exposure of 4- to 5-day-old colostrum-deprived gnotobiotic piglets to the Gottfried strain did not prevent virus shedding or diarrhea after challenge 3 weeks later with the virulent heterotypic OSU strain; however, there was an apparent decrease in duration of virus shedding (Table 2, group 1). Similarly, piglets in group 2 initially exposed to the virulent OSU and challenged 3 weeks later with the virulent Gottfried strain shed virus, although with an apparent decrease in duration, and developed diarrhea. Thus, significant heterotypic protection was not observed. In earlier studies, it was shown that prior infection with OSU or Gottfried completely protected gnotobiotic piglets from diarrhea or virus excretion when challenged 3 weeks later with the homotypic virulent virus (2; data not shown). Challenge of previously uninfeected 25-day-old gnotobiotic piglets with either OSU or Gottfried virus (groups 5 and 6) induced diarrhea of shorter duration than that observed in 4- to 5-day-old piglets (5.5 days versus 9.0 days for OSU and 7.0 days versus 12.0 days for Gottfried); however, the median duration of virus shedding was similar or identical.

**Administration of single-gene substitution OSU × Gottfried reassortant 11-1 to 4- to 5-day-old gnotobiotic piglets induced diarrhea, and the infected piglets shed virus (Table 2, groups 3 and 4).** However, when these piglets were challenged 3 weeks later with virulent OSU, significant diarrhea was not observed (two of four piglets showed a transient mild diarrhea) and there was a delayed onset of virus shedding of short duration (group 3). When animals

<table>
<thead>
<tr>
<th>TABLE 1. Antigenic characterization of a single-gene substitution OSU × Gottfried reassortant 11-1 by PRN assay</th>
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</thead>
<tbody>
<tr>
<td>Porcine rotavirus</td>
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<tr>
<td></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Gottfried</td>
</tr>
<tr>
<td>OSU × Gottfried 11-1</td>
</tr>
<tr>
<td>SB-1A</td>
</tr>
<tr>
<td>OSU</td>
</tr>
</tbody>
</table>

a Values derived from one test. Homologous titers are in boldface type.
TABLE 2. Immunity to challenge with parental porcine rotaviruses OSU and Gottfried induced by the reassortant rotavirus 11-1 (Gottfried VP7, OSU all other genes) in gnotobiotic piglets

<table>
<thead>
<tr>
<th>Gnotobiotic piglet group (no.)</th>
<th>Rotavirus administered</th>
<th>Primary infection (at age 4 to 5 days)</th>
<th>Challenge (3 weeks after primary infection)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Duration (median days) of:</td>
<td>Rotavirus administered</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Virus shedding</td>
<td>Diarrhea</td>
</tr>
<tr>
<td>1 (4)</td>
<td>Gottfried</td>
<td>7.5</td>
<td>12.0</td>
</tr>
<tr>
<td>2 (4)</td>
<td>OSU</td>
<td>10.0</td>
<td>9.0</td>
</tr>
<tr>
<td>3 (4)</td>
<td>OSU × Gottfried 11-1</td>
<td>7.75</td>
<td>7.25</td>
</tr>
<tr>
<td>4 (4)</td>
<td>OSU × Gottfried 11-1</td>
<td>7.75</td>
<td>7.75</td>
</tr>
<tr>
<td>5 (2)</td>
<td>none</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6 (2)c</td>
<td>none</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Two of four piglets showed a transient mild diarrhea.
A NA, Not applicable.
C One piglet died on day 7 (virus shedding and diarrhea occurred from day 1 to day 6).

Previously infected with the OSU × Gottfried 11-1 reassortant were challenged with virulent Gottfried, neither virus shedding nor diarrhea was observed (group 4).

Neutralizing antibody response of gnotobiotic piglets initially treated with OSU × Gottfried reassortant 11-1 rotavirus and challenged 3 weeks later with either virulent OSU or Gottfried. Exposure of 4- to 5-day-old piglets to single-gene substitution OSU × Gottfried reassortant 11-1 induced a significant antibody response to both Gottfried and OSU viruses (Table 3, groups 3 and 4). Of note is the finding that the neutralizing antibody titer induced by OSU VP3 is higher than that induced by the VP7 of Gottfried. The level of serum neutralizing antibodies to OSU VP3 as determined with OSU virus (VP3:5; VP7:5) was higher than when a single-gene substitution OSU × DS-1 reassortant 46-1, which derived only the VP3 gene from its OSU parent and the remaining 10 genes from human DS-1 virus (VP3:5; VP7:2), was used for assay.

Sera obtained 16 days after OSU virus challenge of group 3 piglets showed a fourfold or greater increase in serum antibody to homologous virus but not to heterologous Gottfried virus. Challenge of group 4 piglets with Gottfried virus induced an increase in neutralizing antibodies which approached fourfold, although a significant increase of neutralizing antibody to heterologous OSU virus was not observed. Also, the serologic response of group 2 piglets suggested that neutralizing antibodies to VP3 of serotype 5 develop slowly and require 37 days or more to reach maximum titers.

**DISCUSSION**

Recent studies have established that the neutralization specificities on VP3, gene product 4, and VP7, gene 8 or 9 product, segregate independently in nature and that VP3 is as potent an immunogen as VP7 in inducing neutralizing antibodies under conditions of parenteral or oral hyperimmunization. Also, it has been shown that mouse dams hyperimmunized orally with a reassortant containing VP3 and VP7 from two distinct rotavirus serotypes protect their pups passively against diarrhea induced by either parental rotavirus (25). Critical questions remaining unanswered were (i) how do the rotavirus outer capsid proteins VP3 and VP7 behave antigenically in the host during a single enteric infection, (ii) to what extent are antibodies induced against VP3 and VP7 involved in resistance against rotavirus disease, and (iii) how effective is a single enteric infection by a reassortant rotavirus in providing resistance to both parental viruses? We addressed these questions in this study by using a homologous system of colostrum-deprived, newborn gnotobiotic pigs free of maternally antibodies and a single VP7 gene substitution porcine rotavirus reassortant.

First, we analyzed the immunogenicity in guinea pigs of the single-gene substitution OSU × Gottfried rotavirus reassortant 11-1, which derived 10 gene segments from the OSU virus (serotype 5) and only the VP7 gene segment (gene 9) from the Gottfried virus (serotype 4). When hyperimmunized parenterally with reassortant 11-1, guinea pigs produced high levels of neutralizing antibodies not only to OSU virus but also to OSU virus (Table 1). Since Gottfried is antigenically distinct from OSU, neutralization of OSU virus by OSU × Gottfried reassortant 11-1 antiserum is due to OSU VP3 antibodies, and neutralization of Gottfried virus by the same antiserum is due to Gottfried VP7 antibodies. This confirms that VP3 is as potent in stimulating neutralizing antibodies as VP7 after parenteral hyperimmunization (11, 12, 24, 25).

Next, we analyzed serum samples obtained from colostrum-deprived, newborn gnotobiotic pigs infected orally with the single-gene substitution reassortant 11-1 and observed that VP3 was as potent an immunogen as VP7 in inducing neutralizing antibodies after enteric infection. That is, the immune system of the host recognizes both VP3 and VP7 equally as judged by induction of equal levels of neutralizing antibodies by the two rotavirus outer capsid proteins (Table 3). It is of interest that the VP3 of a reassortant rotavirus such as 11-1 may be more effective than its parental rotavirus in inducing neutralizing antibodies not only under conditions of hyperimmunization reported previously (11, 12, 24, 25) but also under conditions of a single oral immunization (this study). In addition, such antibodies were associated with resistance to infection, disease, or disease and infection caused by the virulent parental rotavirus used to derive the reassortant. Neutralizing antibodies induced by the Gottfried VP7 of the OSU × Gottfried reassortant 11-1 were associated with resistance of piglets to challenge with virulent Gottfried virus, as indicated by absence of diarrhea and virus shedding. Similarly, antibodies to OSU VP3 of the reassortant 11-1 were associated with protection of piglets from diarrhea (two of four showed a transient, mild diarrhea) upon challenge with virulent OSU virus; virus shedding was also delayed in onset and was decreased in duration (Table 2). These observations indicate that a single oral administration of a reassortant
TABLE 3. Neutralizing antibody response of guinea pig and swine to reassortant rotavirus vaccines.

<table>
<thead>
<tr>
<th>Group</th>
<th>Challenge</th>
<th>Primary virus</th>
<th>Challenge virus</th>
<th>VP3</th>
<th>VP7</th>
<th>VP4</th>
<th>VP2</th>
<th>VP3-VP7</th>
<th>VP2-VP4</th>
<th>VP3-VP4</th>
<th>VP7-VP4</th>
<th>VP2-VP7</th>
<th>VP3-VP2</th>
<th>VP7-VP3</th>
<th>VP4-VP3</th>
<th>VP7-VP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSU</td>
<td>DS-1A</td>
<td>VP3-VP7</td>
<td>VP3-VP7</td>
<td>YES</td>
<td>YES</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>OSU</td>
<td>DS-1A</td>
<td>VP7-VP3</td>
<td>NO</td>
<td>NO</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>OSU</td>
<td>DS-1A</td>
<td>VP2-VP4</td>
<td>NO</td>
<td>NO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>OSU</td>
<td>DS-1A</td>
<td>VP4-VP3</td>
<td>NO</td>
<td>NO</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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Note: OSU virus and then challenged 7 days later with OSU or reassortant (100 days). Mann-Whitney U test showed that the observed differences between the two groups were significant (0.01).
ACKNOWLEDGMENTS
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LITERATURE CITED