Viral Determinants of Rotavirus Pathogenicity in Pigs: Evidence that the Fourth Gene of a Porcine Rotavirus Confers Diarrhea in the Homologous Host

JANICE C. BRIDGER, GABRIELE I. TAUSCHER, AND ULRICH DESSELBERGER

Department of Pathology and Infectious Diseases, The Royal Veterinary College, University of London, London NW1 0TU, and Clinical Microbiology and Public Health Laboratory and Division of Virology, Department of Pathology, University of Cambridge, Addenbrooke’s Hospital, Cambridge CB2 2QW, United Kingdom

Received 26 January 1998/Accepted 4 May 1998

A porcine rotavirus (prv) monoreassortant, S-F4, which carries RNA segment 4 of the pig-pathogenic variant prv 4F in the genetic background of the pig-apathogenic variant prv 4S (G. I. Tauscher and U. Desselberger, J. Virol. 71:853–857, 1997), was found to be pathogenic in gnotobiotic piglets. This indicates that RNA segment 4 of the pig-pathogenic variant prv 4F is a major determinant of pathogenicity in its homologous host.

Rotaviruses are the main cause of viral gastroenteritis in infants and young children worldwide and are responsible for approximately 20% of diarrhea-associated deaths in children under five in developing countries (8, 14). Rotaviruses are also a major pathogen of farm animals (2) and were identified as the most common enteropathogen associated with outbreaks of diarrhea in calves (17, 18).

Rotavirus structure, classification, gene composition, and replication have been well clarified (reviewed in reference 9); however, other aspects of rotavirus behavior, for example, the true correlates of protection or factors determining pathogenicity, remain less clear despite extensive research (reviewed in references 6 and 15).

The present study investigated the influence of RNA segment 4 of rotavirus on pathogenicity in the piglet model. This model was chosen because a pair of porcine rotavirus (prv) variants, termed prv 4F and 4S, and reassortants thereof were available. Moreover, piglets are the only animal host in which infection with human rotavirus causes diarrhea (20, 21).

The two prv variants possess identical electrophoretic migration profiles of the 11 segments of genomic double-stranded RNA on polyacrylamide gels with the exception of RNA segment 4 (RNA 4). RNA 4 of variant prv 4F (fast-migrating gene 4) migrated ahead of that of the variant prv 4S (slow-migrating gene 4) (11). The two variants differ significantly in their in vitro growth parameters: variant 4S grows to high titers in cell culture after prolonged incubation. Variant prv 4F is pathogenic in gnotobiotic piglets, causing severe diarrhea and weight loss, whereas variant prv 4S remains apathogenic while replicating to comparable titers (4).

Sequence analysis of the two RNAs 4 revealed a nucleotide identity in corresponding positions of only 68% and a predicted amino acid identity of only 71% (7). By contrast, RNA segments 5, 6, and 8 of the two variants (coding for NS53, VP6, and VP7, respectively) were found to be virtually identical (7). These results suggested that the two viruses are genetically related by a reassortment event. However, without sequencing of all the genes of both variants, evidence for the involvement of RNA 4 in pathogenesis remained circumstantial. Hence, we produced a monoreassortant, termed prv S-F4, which carries RNA 4 of the pathogenic prv 4F variant in the genetic background of the other 10 segments of the pig-apathogenic prv 4S variant, as described in detail previously (19). Here we report the outcome of inoculation of gnotobiotic piglets with the monoreassortant S-F4 and both parental viruses in terms of virus replication and pathogenicity.

The origin and biological characteristics of the parent prv variants 4F and 4S and of the monoreassortant S-F4 were described previously (4, 11, 19). Infectivity titers of inocula were determined by titration in 10-fold serial dilutions in MA104 cells grown in microtiter trays. After overnight incubation, rotavirus was detected by immunoperoxidase staining with a bovine antiseraum to the UK bovine rotavirus. The numbers of foci of infected cells were counted at suitable dilutions, and titers were expressed as focus-forming units (FFU) per milliliter. Infectivity titers in fecal samples were determined by titration in 10-fold serial dilutions, using five roller tube cultures of MA104 cells per dilution. Cytopathic effect was evaluated after 4 days, and titers were expressed as log10 50% tissue culture infective doses (TCID50) per milliliter of feces. Rotavirus RNA was extracted from feces and detected after separation by polyacrylamide gel electrophoresis (PAGE) and silver staining as described previously (19).

Gnotobiotic piglets were derived by hysterotomy and then housed in pairs in positive-pressure isolators. They were reared on a milk-based diet and allocated randomly to the different treatment groups. They were inoculated at 5 or 6 days of age with the monoreassortant rotavirus S-F4 or the parent prv 4F or prv 4S, or they were sham inoculated at the doses shown in Table 1. Three serial pig passages were conducted in three litters, with bacterium-free fecal suspensions, because serial pig passage was required previously to reveal the pathogenicity of prv 4F after cell culture passage (4). The first pig passage was conducted with viruses which had been passaged seven times (4S), eight times (4F), or seven times (S-F4) in cell culture. The infectivity titers of the doses administered were rechecked by transfer of aliquots from the isolator after piglet inoculation. Piglets were monitored for clinical signs of infection (diarrhea, reduced food intake, dehydration, weight loss, and depression) from 2 days before to 10 days after inoculation. Fecal samples were collected daily, and cord blood was sampled to determine preinfection antibody status. Diarrhea...
FIG. 1. Daily weights (in kilograms) of representative piglets inoculated with S-F4 (■), prv 4F (●), and prv 4S (○), and of a sham-inoculated piglet (△), at the third serial passage of the viruses.

was defined as production of light-colored (cream to light yellow) feces often with a curdled or floccular appearance.

The ability to cause disease was transferred with RNA 4 of the pathogenic variant prv 4F to the monoreassortant S-F4. Five of the six piglets inoculated with S-F4 during the three serial pig-to-pig passages developed diarrhea which commenced between days 1 and 3 after inoculation at the second and third pig passages. All four piglets at the second and third pig passages failed to gain weight from day 1 or 2 after inoculation to day 3 or 5 after inoculation (Fig. 1). They lost, on average, 11% of their body weight. One of the two piglets at the first pig passage failed to develop clinical signs, but the second developed diarrhea between days 4 and 5 postinoculation. Five of the six piglets became visibly depressed, and four piglets, at passages 1, 2, and 3, required rehydration therapy. By contrast, diarrhea was not observed in the four piglets which had received prv 4S (Table 1). The differences between S-F4- and prv 4S-inoculated piglets were statistically significant in the numbers of days with diarrhea and with failure to gain weight; the numbers of days with depression and that treatment was required were greater in animals inoculated with S-F4, but not significantly so. The severity of infection with the reassortant S-F4 was similar to that with the parent prv 4F (Table 1); the numbers of days that pigs had diarrhea, failed to gain weight, had depression, and required treatment were not statistically different.

The findings of an earlier study (4), which showed a clear difference in pathogenicity between the parent prv 4F and prv 4S, were confirmed with additional piglets in the present study. As before, there were significant differences in the numbers of days with diarrhea (P < 0.01) and with failure to gain weight (P < 0.05) (Table 1; results of chi-square testing not shown). There was no progression to more severe clinical signs during the three serial pig-to-pig passages of prv 4F or S-F4 (data not shown). There was no statistical difference in the number of days with failure to gain weight between animals infected with prv 4S and sham-inoculated piglets.

RNA profile analysis by PAGE showed that viral replication took place in all virus-infected piglets starting between days 1 and 3 after inoculation. All piglets excreted rotaviruses with the expected RNA profiles. There was a clear relationship between subjective readings of the intensities of RNA patterns on gels and infectivity titers which were determined for selected samples (Table 2). There were no statistically significant differences in the mean peak infectivity titers among the three virus-infected groups (Table 1).

Thus, the data presented here demonstrated clearly that the monoreassortant, S-F4, carrying the RNA 4 of the pathogenic variant prv 4F in the genetic background of 10 segments of the apathogenic variant prv 4S, was pathogenic. The data also confirmed the differences in pathogenicity between prv 4F and prv 4S reported previously (4).

Several rotavirus genes have been implicated in rotavirus pathogenicity, but the genes responsible appear to differ in different animal models (1, 5, 6, 10, 13, 16). RNA segments 3, 4, 5, 7, 8, and 10 have been shown to be involved. The data acquired by Hoshino et al. (13) in the piglet model are particularly relevant to the present study. These authors showed that monoreassortants constructed with a pig-pathogenic prv and a nonpathogenic prv were pathogenic, whereas a monoreassortant with the pig-pathogenic prv and a pig-then-human-type 1 segment 4 was nonpathogenic. The present study confirmed the differing pathogenicity of RNA 4 of different strain backgrounds of S-F4, prv 4F, and prv 4S.

**TABLE 1. Clinical signs and virus excretion during three serial pig passages of the reassortant S-F4, prv 4F, and prv 4S**

<table>
<thead>
<tr>
<th>Virus inoculum&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of pigs&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. of days with parameter/no. of observation days&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Peak log&lt;sub&gt;10&lt;/sub&gt; TCID&lt;sub&gt;50&lt;/sub&gt;/ml of feces (mean ± SD)</th>
<th>Mean first day of virus excretion&lt;sup&gt;d&lt;/sup&gt; (range)</th>
<th>Mean duration of virus excretion&lt;sup&gt;d&lt;/sup&gt; (range) (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4F</td>
<td>6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Diarrhea: 13/56, Failure to gain weight: 10/40, Depression: 6/60, Treatment given: 2/60, Virus in feces: 26/52</td>
<td>6.4 ± 0.8</td>
<td>1.6 (1–2)</td>
<td>4.8 (2–7)</td>
</tr>
<tr>
<td>S-F4</td>
<td>6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Diarrhea: 24/60, Failure to gain weight: 13/40, Depression: 8/60, Treatment given: 7/60, Virus in feces: 29/60</td>
<td>6.8 ± 0.6</td>
<td>1.7 (1–3)</td>
<td>4.8 (3–7)</td>
</tr>
<tr>
<td>4S</td>
<td>4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Diarrhea: 14/60&lt;sup&gt;f&lt;/sup&gt;, Failure to gain weight: 3/40&lt;sup&gt;f&lt;/sup&gt;, Depression: 0/40, Treatment given: 0/40, Virus in feces: 12/20&lt;sup&gt;f&lt;/sup&gt;</td>
<td>6.9 ± 0.6&lt;sup&gt;g&lt;/sup&gt;</td>
<td>1&lt;sup&gt;h&lt;/sup&gt; (1)</td>
<td>6&lt;sup&gt;h&lt;/sup&gt; (5–7)</td>
</tr>
<tr>
<td>Sham</td>
<td>3</td>
<td>Failure to gain weight: 0/30, Depression: 2/30, Treatment given: 0/30, Virus in feces: NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

<sup>a</sup> The doses given at the first, second, and third serial pig passages, respectively, were as follows: for 4F, 2.4 × 10⁶, 1.1 × 10⁶, and 3.3 × 10⁶ FFU; for S-F4, 1.3 × 10⁶, 5.9 × 10⁵, and 4.4 × 10⁵ FFU; for 4S, 4.5 × 10⁶, 5.3 × 10⁵, and 3.0 × 10⁵ FFU. Sham-inoculated pigs were inoculated with 1.0 ml of cell culture fluid.

<sup>b</sup> The mean weights of piglets (range) in the four groups on day 0 were 1.7 (1.25 to 2.3) kg for 4F, 1.3 (0.7 to 1.8) kg for S-F4, 1.4 (0.7 to 1.8) kg for 4S, and 1.1 (0.9 to 1.3) kg for the sham-inoculated group.

<sup>c</sup> Adjacent values in a column are not significantly different (P > 0.05; chi-square test) unless otherwise noted.

<sup>d</sup> Detected by PAGE.

<sup>e</sup> Feces were not collected from two pigs for 2 days during the observation period of the first passage.

<sup>f</sup> The doses given at the second and third pig passages were 6.3 × 10⁶, 3.3 × 10⁶, and 2.4 × 10⁶ FFU for 4F, 1.3 × 10⁶, 1.1 × 10⁶, and 2.4 × 10⁵ FFU for S-F4, and 3.3 × 10⁵, 5.9 × 10⁴, and 4.4 × 10⁴ FFU for 4S. Sham-inoculated pigs were inoculated with 1.0 ml cell culture fluid.

<sup>g</sup> Weights were not determined at the first passage for 4F and S-F4.

<sup>h</sup> The doses given at the second and third pig passages were 6.3 × 10⁶, 3.3 × 10⁶, and 2.4 × 10⁶ FFU for 4F, 1.3 × 10⁶, 1.1 × 10⁶, and 2.4 × 10⁵ FFU for S-F4, and 3.3 × 10⁵, 5.9 × 10⁴, and 4.4 × 10⁴ FFU for 4S. Sham-inoculated pigs were inoculated with 1.0 ml cell culture fluid.

<sup>i</sup> Data from two pigs at the third serial pig passage.

<sup>j</sup> NT, not tested.
pig-apathogenic human rotavirus were apathogenic and failed to replicate productively when RNA segments 3, 4, 8 (which codes for VP7), and 10 of the pathogenic virus had been replaced individually by the corresponding segment of the apathogenic virus. Conversely, reassortants of the apathogenic human virus carrying one to three of the above-cited genes of the pathogenic virus did not replicate productively and were not pathogenic. However, a tetrareassortant in which all four RNA segments (segments 3, 4, 9 [which codes for VP7], and 10) of the apathogenic virus were replaced by those of the pathogenic virus possessed the ability to replicate in piglets, and pathogenicity was restored. These data differ from ours in that the monoreassortant, S-F4, carrying RNA 4 from a pig-pathogenic rotavirus was pathogenic. The difference may be due to the fact that in the experiments of Hoshino et al. (13) the apathogenic virus was a human virus which failed to replicate in piglets, whereas the apathogenic pig virus used in our studies replicates well in piglets but does not cause disease (4). In our study no attempt was made to identify the gene(s) which allows full replication in piglets without causing diarrhea.

RNA segment 10 has been shown to act as a viral enterotoxin in mice (1). The question of a possible involvement of RNA segment 10 in the pig model remains to be answered. RNA segments 10 of prv 4F and prv 4S have not been sequenced, but experiments reported in the present paper suggest that this segment may be less important than RNA 4 in this virus-host relationship. In reassortment experiments some of us reported previously (19), a monoreassortant, B-F10, which possesses RNA segment 10 of prv 4F in the background of the genome of the bovine rotavirus, which is apathogenic in piglets (3), was obtained. It would be relevant to test this reassortant for pathogenicity in piglets in parallel with other available reassortants of different genetic compositions (e.g., B-F4, B-F4,5, and B-F4,5,8 [19]) to establish the influence of coassortment of different genes of a pathogenic virus into an apathogenic virus on the ability to replicate in pigs and the ability to cause disease. The mechanism by which RNA 4 influences virulence is not known, but a tendency toward earlier virus excretion after inoculation has been noticed with the virulent variant prv 4F (4). The mechanism of rotavirus virulence has been studied in more detail in calves; like the rotavirus investigated in the present study, bovine rotaviruses which differed in their abilities to cause diarrhea were excreted at similar levels when assayed in cell culture, although the onset of virus excretion occurred sooner in clinically affected calves (2a, 3a).

Differences in the site of replication in the small intestine, the area of small intestinal epithelium infected, and the ability to damage enterocytes were found between a virulent and an avirulent bovine rotavirus in a study of the pathology of rotavirus virulence (12). It remains to be established if these parameters account for the differences in virulence between prv 4F and prv 4S, but the results presented here greatly strengthen the argument for RNA 4 as a major determinant for rotavirus pathogenicity in the piglet model.

We thank Lynn Dorsett, Ian Bailey, and Dennis Harris for care of the animals and collection of data and samples. J.C.B. acknowledges the encouragement of Colin R. Howard.

The study was supported by a grant from the Wellcome Trust to J.C.B., by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft, Germany (grant TA100-80-1-1), to G.I.T., and by a grant from the Isaac Newton Trust, Cambridge, United Kingdom.

REFERENCES


TABLE 2. Infectivity titers of fecal samples differing in intensity of rotavirus RNA profile determined by PAGEa

<table>
<thead>
<tr>
<th>Intensity of rotavirus RNA profileb</th>
<th>Infectivity titera (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>3.9 ± 0.8 (7)</td>
</tr>
<tr>
<td>+</td>
<td>4.3 ± 1.3 (4)</td>
</tr>
<tr>
<td>+ +</td>
<td>5.1 ± 0.7 (16)</td>
</tr>
<tr>
<td>+ + +</td>
<td>6.5 ± 0.7 (32)</td>
</tr>
</tbody>
</table>

a RNA intensity values and viral infectivity titers of all three viruses were included in the analysis.

b −, no visible RNA segments; +, trace of segments; + +, clearly visible segments; + + +, intensely visible segments.

*Expressed as log_{10} TCID_{50} per milliliter of feces (arithmetic mean ± standard deviation).