Pathogenesis of an attenuated and a virulent strain of group A human rotavirus in neonatal gnotobiotic pigs

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Gnotobiotic (Gn) pigs were orally inoculated with Wa strain (G1P1A[P8]) human rotavirus (Wa HRV) serially passaged in Gn pigs (virulent) or cell culture (attenuated) to determine the median virus infectious dose (ID₅₀) and to assess the site of infection and type and progression of morphological lesions and clinical responses induced by these two strains in Gn pigs. The ID₅₀ of virulent Wa HRV was ≤ 1 f.f.u. whereas the infectivity of attenuated Wa HRV had to be determined by seroconversion and was ~ 1·3 × 10⁶ f.f.u. Diarrhoea developed at 13 h post-inoculation (p.i.) in pigs inoculated with ~ 10⁵ f.f.u. of virulent Wa HRV and correlated with the presence of viral antigen within villous epithelial cells; villous atrophy developed later at 24 h p.i. and correlated with peak faecal viral titres; recovery from disease correlated with the return of morphologically normal villi. Virus, diarrhoea and villous atrophy were not detected in pigs inoculated with ~ 2 × 10⁶ f.f.u. attenuated Wa HRV although HRV-specific serum antibodies were present by 7 days p.i. These findings demonstrate that virulent Wa HRV infection in Gn pigs occurs primarily within intestinal villous epithelial cells with villous atrophy developing as a sequela to infection. However, factors other than villous atrophy appear to contribute to the early stages of HRV-associated disease expression in Gn pigs. The ability of the attenuated virus to elicit virus-neutralizing serum antibodies without disease or pathology indicates promise in the use of such strains for oral immunization.

Introduction

Rotaviruses have been classified into seven distinct groups (A–G), based on serological relationships and electropherotypes, with the group A rotaviruses being the most widespread among humans and animals (Conner et al., 1994; Saif, 1990a; Theil, 1990). The group A rotaviruses cause mild asymptomatic infection to severe life-threatening diarrhoea in susceptible hosts (Greenberg et al., 1994; Saif, 1990b). Variations exist in prevalence and virulence among the group A rotaviruses which may account for the spectrum of disease observed among natural hosts (Collins et al., 1989; Greenberg et al., 1994; Hall et al., 1976; Theil, 1990).

Rotaviruses are generally considered to be host-specific although under experimental conditions several animal species have been infected with rotaviruses from heterologous species origin (Greenberg et al., 1994; Gouvea et al., 1986; Hall et al., 1976; Schaller et al., 1992; Steel & Torres-Medina, 1984; Wyatt et al., 1980). The experimental infection of a species with a rotavirus of heterologous species origin is not surprising in view of recent phylogenetic studies that suggest interspecies infections probably occur naturally (Taniguchi et al., 1994). Neonatal mice and gnotobiotic calves and pigs are reported to be susceptible to infection and disease following inoculation with rotavirus strains of human origin (Gouvea et al., 1986; Mebus et al., 1977; Torres-Medina et al., 1976). However, detailed pathogenesis studies of HRV infection in animals are few and limited to virulent HRV strains (Gouvea et al., 1986; Mebus et al., 1977). The reported pathological lesions induced by virulent strains of HRV in animals are similar to those induced by virulent rotaviruses of homologous host origin and include the loss of intestinal absorptive cells and villous atrophy followed by crypt hyperplasia and mucosal epithelium repair (Collins et al., 1989; Hall et al., 1993; Theil et al., 1978; McAdaragh et al., 1980; Castrucci et al., 1983; Snodgrass et al., 1977; Adams & Kraft, 1967; Pappenheimer & Enders, 1947; Hall et al., 1976; Pearson & McNulty, 1977; Crouch & Woode, 1978).

The gnotobiotic (Gn) pig has been used by our laboratory to study rotaviral pathogenesis and the development of immunity to homologous and heterologous rotavirus infec-
tions (Chen et al., 1995; Theil et al., 1978, 1985; Schaller et al., 1992; Wyatt et al., 1980). In collaboration with researchers at the National Institutes of Health (Bethesda, Maryland, USA), our laboratory adapted a human rotavirus (Wa strain, G1P[1A][P8]); isolated from a symptomatically infected human infant) to cell culture (Wyatt et al., 1980). The G serotype (G1) of Wa HRV represents the most common serotype associated with rotaviral infections in infants and children (Theil, 1990; Conner et al., 1994; Saif, 1990a). The wild-type Wa HRV also proved virulent (induced disease) to newborn Gn pigs (L. J. Saif, unpublished results), but after adaptation to cell culture and multiple cell culture passages, the virus was attenuated (did not induce disease) for the Gn pig (L. A. Ward, B. I. Rosen, & L. J. Saif, unpublished results). Thus, the objective of the present study was to examine the sites of viral infection by these two Wa HRV strains in the Gn pig and to correlate these findings with the development of morphological lesions (if any) and clinical responses.

Methods

■ Virus stocks and cells. An infant stool specimen containing Wa HRV (from R. G. Wyatt, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland) was used to orally inoculate Gn pigs whose intestinal contents were collected and serially passaged (orally) in additional pigs. Pooled intestinal contents obtained from the 16th Gn pig passage was used as the virulent Wa HRV stock inoculum. After 11 Gn pig passages, the Wa HRV was adapted to culture in fetal Rhesus monkey kidney (MA104) cells and plaque purified (Wyatt et al., 1980). The plaque-purified, cell culture-adapted Wa HRV was subsequently cloned six times in MA104 cells by limiting dilution. An MA104 cell culture lysate containing the 27th passage of the cloned cell culture-adapted Wa HRV was used as the attenuated Wa HRV stock inoculum. The titre of each inoculum was determined by cell culture immunofluorescence (CCIF) assay (Bohl et al., 1982).

To confirm that multiple passages in either Gn pigs or cell culture did not alter the electropherotype of Wa HRV, phenol–chloroform extracts of double-stranded RNA from both Wa HRV inocula were prepared for PAGE analysis as described (Theil et al., 1981). An extract of Gottfried porcine rotavirus was included as a group A rotavirus reference standard. Bands were visualized by silver staining (Silver Stain Kit; Bio-Rad).

■ Animals and experimental design. Near-term piglets were derived and maintained under germ-free conditions as described (Meyer et al., 1964). A total of 45 Gn pigs were used for determining the infectivity of the Wa HRV stock inocula in Gn pigs. Each Wa HRV inoculum was serially diluted in MEM (~ 10⁶ to 10¹ f.f.u./ml of virulent Wa HRV and ~ 10⁴ to 10² f.f.u./ml of attenuated Wa HRV). Diluted suspensions were fed as 5 ml aliquots to 4- to 5-day-old Gn pigs 10 min after a 5 ml feeding of 100 mM-sodium bicarbonate (Graham et al., 1987). Pigs were examined daily for diarrhoea and their faeces scored as follows: 0, normal; 1, pasty; 2, semi-liquid; and 3, liquid. Diarrhoea was present if the faecal consistency score was ≥ 2. Rectal swabs were collected daily and blood samples drawn weekly. Infectivity was defined as the dose of live virulent or attenuated Wa HRV required to infect 50% of inoculated pigs as determined by faecal virus shedding (median infectious dose, ID₅₀) or seroconversion at 21 days p.i. (median seroconversion dose, SD₅₀), respectively.

For morphological studies, a total of 30 four-day-old Gn pigs were orally inoculated with ~ 2 x 10⁸ f.f.u. (~ 10⁵ ID₅₀) attenuated Wa HRV (14 pigs) or ~ 10⁶ f.f.u. (~ 10⁴ ID₅₀) virulent Wa HRV (16 pigs); 6 age-matched pigs were inoculated with diluent (MEM) as controls. Two pigs from each Wa HRV-inoculated group were euthanized at 13 h p.i. and 1 control and 2–4 Wa HRV-inoculated pigs were euthanized at 24, 48, 72 and 96 h p.i. and 7 days p.i. Blood was collected from each pig at inoculation and euthanasia. Rectal swabs were taken daily until euthanasia and each pig’s faecal consistency scored 0–3 as described above. Intestinal tracts were removed from the abdominal cavities at euthanasia and contents of the small intestine and caecum/spiral colon collected by aspiration with a syringe and needle. Fresh specimens of duodenum, jejunum, ileum, mesenteric lymph nodes (MLN), stomach, colon, rectum, liver, spleen and each kidney were collected and placed on ice for preparation of impression smears. Nine 6 cm-long sections of small intestine at equally spaced sites beginning 5 cm distal to the pylorus and ending 5 cm proximal to the ileo-caecal junction were excised from each intestinal tract and immediately immersed in 10% buffered zinc formalin. Portions of the pyloric stomach, spiral colon, rectum, MLN, palatine tonsils, nasal turbinates, adrenals, spleen, liver, kidneys, heart, lung and brain were also excised and immediately placed in fixative.

■ Gross and microscopic evaluation of tissues. All organs were examined in situ before removal from the body cavity. The stomach and intestinal fill (distension), consistency of ingesta, thicknesses of the intestinal walls, size and fill of the caecum/spiral colon, size and colour of MLN, and the presence or absence of chyle within mesenteric lymphatics were noted and recorded. Of the nine small intestinal segments from each pig placed in fixative, the first two segments were considered to be duodenum, the middle four segments, jejunum and the last three segments, ileum. After at least 48 h in fixative, one segment from each region of each pig’s small intestine was opened longitudinally and examined for villous atrophy with the aid of a dissecting microscope. Villous atrophy was considered to be sparse if multiple pinpoint depressions were present in one region of the small intestine; scattered if multiple depressions were seen in two regions; and widespread if point depressions coalesced and were seen in all regions. Intestinal segments were then cross-sectioned at 0.5 cm intervals and processed by dehydration in graded ethanols and embedded in paraffin wax. Sections of each additional tissue collected were similarly prepared. Sections (4 µm) of the paraffin-embedded tissues were made and stained with Mayer’s haematoxylin and eosin. Twelve non-replicate sections of each small intestinal region from at least two Wa HRV-inoculated pigs were examined microscopically at each time point. Histological evaluation was done on coded samples and a comparison made with tissues from age-matched controls.

■ Detection of Wa HRV in tissues. A direct fluorescent antibody (FA) assay was used to evaluate the distribution of Wa HRV antigens in impression smears prepared from the fresh specimens of duodenum, jejunum, ileum, MLN, stomach, colon, rectum, liver, spleen and kidneys collected at necropsy. Preparation, staining and examination of the smears were done as previously described (Bohl et al., 1982). Cells were visualized by fluorescent microscopy and counted with the aid of an optical grid. A minimum of 1000 total cells per smear was visualized for determination of the percentage of fluorescent cells.

■ Detection of Wa HRV in faeces

(i) Cell-culture immunofluorescent (CCIF) assay. The CCIF assay was done on 96-well microtitre plates as previously described on coded samples to determine the titre of infectious HRV in inocula and faeces or intestinal contents (Bohl et al., 1982). Fluorescing cells were visualized by fluorescent microscopy and the number of fluorescent focus-forming units (f.f.u.) counted in each well.
(ii) Immune electron microscopy (IEM). The contents of the small intestine and caecum/spiral colon collected from 1 pig from each Wa HRV-inoculated group at 13, 24, 48, 72, and 96 h p.i. and from 2 control pigs at 24 and 48 h p.i. were examined by IEM using described methods (Saif et al., 1977) to detect HRV and evaluate virus morphology.

Detection of Wa HRV-specific antibodies in serum

(i) Indirect immunofluorescent assay (IFA). Coded serum samples from the Wa HRV-inoculated pigs were evaluated by IFA on acetone-fixed Wa HRV-infected MA104 cell monolayers (Kang et al., 1989). Fourfold dilutions of each serum sample were incubated for 30 min at 37°C, in duplicate wells containing acetone-fixed (attenuated) Wa HRV-infected MA104 cell monolayer. Serum samples from the age-matched controls served as negative controls. Bound serum antibodies were detected by use of a FITC-conjugated rabbit anti-swine IgG hyperimmune serum prepared as described (Theil et al., 1985). This IFA permitted the detection of antibodies binding to structural and non-structural Wa HRV proteins in serum samples. The IFA titre was expressed as the reciprocal of the highest serum dilution producing specific immunofluorescence.

(ii) Plaque reduction virus neutralization (VN) antibody assays. The plaque reduction VN antibody assay was done as described on coded serum samples using attenuated Wa HRV as the titrating virus (Schaller et al., 1992). Serum VN antibody titres were calculated as the reciprocal of the serum dilution that reduced the number of plaques by ≥ 80%; serum VN titres of < 4 were considered to be negative and assigned a value of 2 for determining the geometric mean titre (GMT).

Results

PAGE analysis of the Wa HRV stock inocula

The results of the PAGE analysis of each Wa HRV inoculum are shown in Fig. 1. The migration patterns of the virulent and attenuated Wa HRV double-stranded (ds) RNA segments were identical to the electropherotype first described for the initial isolate of Wa HRV (Bohl et al., 1984). Co-electrophoresis of the two Wa HRV inocula genomes produced 11 distinct co-migrating bands of dsRNA.

Determination of the Wa HRV stock inocula ID50 in Gn pigs

The clinical and serological responses of the Gn pigs used to establish the ID50 of the virulent Wa HRV inoculum are summarized (Table 1). Because all pigs, even those receiving the lowest dose (estimated at < 1 f.f.u.) shed virus after virulent Wa HRV inoculation, the ID50 for the virulent Wa HRV inoculum was considered to be ≤ 1 f.f.u./ml. The median diarrhoeal dose (DD50) of the virulent Wa HRV stock inoculum in Gn pigs was similarly estimated to be ≤ 1 f.f.u./ml since no less than 50% of pigs at any given dose of virulent Wa HRV developed diarrhoea (Table 1).

None of the attenuated Wa HRV-inoculated pigs shed virus and only a single pig inoculated with 2 × 107 f.f.u. of attenuated Wa HRV developed transient loose stools (faecal score 2 for 1 day duration) which was attributed to diet. However, VN serum antibodies were present in 33% (1/3) of pigs inoculated with 8 × 106 f.f.u., 80% (4/5) of pigs inoculated with 4.5 × 107 f.f.u. and 100% (15/15) of pigs inoculated with ≥ 107 f.f.u. Thus, using the method of Reed & Muench (1938) on the seroconversion data, the ID50 for the attenuated Wa HRV inoculum was ~ 1.3 × 105 f.f.u.

Clinical and serological responses of Gn pigs inoculated with Wa HRV

Marked differences were observed between the virulent and attenuated Wa HRV strains in terms of virus shedding and diarrhoea in both studies (infectivity and morphological). The faecal virus shedding and clinical disease of pigs inoculated with various doses of virulent Wa HRV for determination of the ID50 are shown (Table 1). The dose of virulent virus did not appear to influence the peak virus titre shed, duration of virus shedding, severity and duration of diarrhoea or VN serum GMT at 21 days p.i. The onset of diarrhoea occurred by 24 to 72 h p.i. with the latter time observed more frequently at doses ≤ 8.5 × 105 f.f.u. None of the attenuated Wa HRV-inoculated pigs shed virus, even at relatively high inoculation doses (3 × 106 f.f.u.) although one pig inoculated with 5 × 107 f.f.u. developed transient loose stools (faecal score = 2 for 1 day duration). The serological responses of the pigs differed...
Table 1. Infectivity (ID₅₀) of the virulent Wa HRV inoculum for gnotobiotic pigs (4–5 days old)

<table>
<thead>
<tr>
<th>Inoculum (f.f.u.)</th>
<th>Animals</th>
<th>Percentage shedding</th>
<th>Mean onset (days)</th>
<th>Mean duration (days)</th>
<th>Peak titre shed (f.f.u./ml)</th>
<th>Percentage with diarrhoea</th>
<th>Mean onset (days)</th>
<th>Mean cumulative faecal score</th>
<th>Serum VN GMT (day 21 p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1</td>
<td>3</td>
<td>100%</td>
<td>2 (0)*</td>
<td>6 (1-2)</td>
<td>1 x 10⁶</td>
<td>67% (2/3)</td>
<td>3 (0-0)</td>
<td>9 (5-0)</td>
<td>82</td>
</tr>
<tr>
<td>~ 10⁶</td>
<td>2</td>
<td>100%</td>
<td>2 (0)</td>
<td>6 (0-7)</td>
<td>2 x 10⁷</td>
<td>100% (2/2)</td>
<td>2 (0-0)</td>
<td>14 (0-7)</td>
<td>96</td>
</tr>
<tr>
<td>2.5 x 10¹</td>
<td>2</td>
<td>100%</td>
<td>2 (0)</td>
<td>5 (0-0)</td>
<td>2 x 10⁷</td>
<td>50% (1/2)</td>
<td>2 (0-0)</td>
<td>4 (4-2)</td>
<td>84</td>
</tr>
<tr>
<td>5.5 x 10²</td>
<td>4</td>
<td>100%</td>
<td>2 (0)</td>
<td>6 (0-6)</td>
<td>3 x 10⁶</td>
<td>100% (4/4)</td>
<td>3 (1-2)</td>
<td>9 (1-3)</td>
<td>71</td>
</tr>
<tr>
<td>3 x 10³</td>
<td>4</td>
<td>100%</td>
<td>2 (0)</td>
<td>7 (2-2)</td>
<td>9 x 10⁶</td>
<td>100% (4/4)</td>
<td>2 (0-5)</td>
<td>14 (3-1)</td>
<td>94</td>
</tr>
<tr>
<td>8.5 x 10⁴</td>
<td>3</td>
<td>100%</td>
<td>1.3 (0.6)</td>
<td>6 (0-0)</td>
<td>1 x 10⁶</td>
<td>67% (2/3)</td>
<td>1.5 (0.7)</td>
<td>11 (5-7)</td>
<td>45</td>
</tr>
<tr>
<td>3 x 10⁵</td>
<td>2</td>
<td>100%</td>
<td>1.5 (0-7)</td>
<td>5 (0-0)</td>
<td>1 x 10⁷</td>
<td>100% (2/2)</td>
<td>1.5 (0-7)</td>
<td>8 (0-7)</td>
<td>84</td>
</tr>
<tr>
<td>1 x 10⁶</td>
<td>2</td>
<td>100%</td>
<td>1.5 (0-7)</td>
<td>7 (2-1)</td>
<td>2 x 10⁶</td>
<td>100% (2/2)</td>
<td>1.5 (0-7)</td>
<td>10 (7-8)</td>
<td>89</td>
</tr>
</tbody>
</table>

* Determined by CCIF assay.
† Diarrhoea present if daily faecal consistency score ≥ 2; Mean cumulative faecal score = Σ(Daily faecal scores from 1–8 days p.i.)/n.
‡ Numbers in parentheses represent 1 SEM.

between the virus inocula. The responses of the virulent Wa HRV-inoculated pigs were independent of the inoculating dose with all pigs developing by day 21 p.i. VN serum antibodies of similar levels (GMT range from 45 to 96). In contrast, seroconversion was dose dependent in the attenuated Wa HRV-inoculated pigs with 100% seroconversion occurring at doses ≥ 10⁷ f.f.u. The VN serum GMT at day 21 p.i. for each virus inoculation dose were as follows: 4.2 (range < 4 to 19) for pigs receiving 8 x 10⁶ f.f.u.; 32 (range < 4 to 86) for pigs receiving 4.5 x 10⁶ f.f.u.; 62 (range 14 to 86) for pigs receiving 2 x 10⁷; 42 (range 18 to 280) for pigs receiving 7 x 10⁷ f.f.u.; and 69 (range 12 to 256) for pigs receiving 3 x 10⁸ f.f.u. The lowest VN serum antibody titres occurred in pigs receiving the lowest dose of attenuated Wa HRV and the highest VN serum antibody titres occurred in pigs receiving the higher doses; however, because some pigs at each dose had relatively low serum antibody titres at day 21 p.i. and sample sizes were small (n = 3 to 7), the VN serum GMT did not differ significantly between doses.

Fig. 2 illustrates the temporal relationship between the daily faecal score and titre of infectious Wa HRV shed by the Wa HRV-inoculated pigs and control pigs in the morphological
Table 2. Distribution of viral antigens as determined by direct FA on impression smears prepared from the tissues of gnotobiotic pigs at various times post-inoculation with virulent Wa HRV

<table>
<thead>
<tr>
<th>Pigs (n)</th>
<th>Time p.i.</th>
<th>Average no. of cells showing HRV-specific immunofluorescence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stomach</td>
<td>Duodenum</td>
</tr>
<tr>
<td>2</td>
<td>13 h</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>24 h</td>
<td>&lt;1</td>
</tr>
<tr>
<td>4</td>
<td>48 h</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>72 h</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>96 h</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>7 days</td>
<td>0</td>
</tr>
</tbody>
</table>

* Denotes presence of free viral antigen not associated with cells.

studies. Of the pigs inoculated with virulent Wa HRV (~ 10^5 f.f.u.), 100% developed diarrhoea for an average duration of 4 days and 100% shed virus for an average of 3.5 days. The highest titre of virus (2 x 10^7 f.f.u./ml) was shed at 24–48 h p.i. The average daily faecal scores of the attenuated Wa HRV-inoculated pigs (received ~ 2 x 10^5 f.f.u.) were similar to the age-matched sham-inoculated controls (none of which developed clinical disease) and no virus was found in their intestinal contents or faeces as determined by CCIF and IEM analysis. However, all of the Wa HRV-inoculated pigs in this study seroconverted by day 7 p.i. (n = 8) as determined by IFA with serum IFA titres of the attenuated Wa HRV-inoculated pigs ranging from 4 to 16 (GMT = 9.5) and from 8 to 64 (GMT = 19) for virulent Wa HRV-inoculated pigs. However, whereas all pigs seroconverted by IFA, only 50% (2/4) of the attenuated Wa HRV- and 75% (3/4) of the virulent Wa HRV-inoculated pigs had VN serum antibody titres by day 7 p.i.

IEM analysis of intestinal contents from the virulent Wa HRV-inoculated pigs revealed a mixed population of double-layered and triple-layered rotavirus particles with negatively-stained centres (suggests empty virus particles) at 24–48 h p.i. The number of empty and double-layered virus particles decreased markedly relative to the number of triple-layered particles at 72–96 h p.i.

Distribution of HRV antigens in tissues following oral inoculation of Gn pigs with live attenuated or virulent Wa HRV

Viral antigens were not detected in impression smears made from the liver, spleen or kidney from any Wa HRV-inoculated pig or control pig post-inoculation. Consistent with the results of the CCIF assay and IEM analysis, virus antigens were not detected within any gastrointestinal tissues from either the attenuated Wa HRV-inoculated pigs or controls but were readily found within the gastrointestinal tissues of the virulent Wa HRV-inoculated pigs (Table 2). Relatively large amounts of antigen were present throughout the small intestine of the virulent Wa HRV-inoculated pigs at 13 h p.i. with smaller amounts of viral antigens additionally detected in gastric, colonic, rectal and MLN tissues at 24 h p.i. From 48–96 h p.i., viral antigen was again limited to the duodenum, jejunum and ileum although trace amounts of cell-associated and free (non-cell associated) antigen were detected in the...
Fig. 4. Intestinal lesions (a–e) in gnotobiotic pigs following oral inoculation with live virulent Wa strain human rotavirus. Haematoxylin and eosin stain. (a, c–e) Bar marker represents 50 μm, in (b) bar marker represents 20 μm. (a) Jejunum 13 h p.i.; absorptive cells separated from basement membranes at villi tips and loss of absorptive cell vacuolation. (b) Jejunum 13 h p.i.; higher magnification of villus tip absorptive cells demonstrating loss of cytoplasmic vacuolation. (c) Jejunum 24 h p.i.; villous atrophy. (d) Ileum 72 h p.i.; villous atrophy, crypt hyperplasia and hyperplasia of Peyer’s patches. (e) Ileum 7 days p.i.; elongated villi covered by vacuolated absorptive cells and prominent submucosal lymphoid follicles. (f) Diluent control (ileum) 7 days p.i. Note small size of submucosal lymphoid follicles. Bar marker represents 50 μm.
Table 3. Correlation of clinical disease, morphological lesions and distribution of HRV antigens within the small intestine of virulent Wa HRV-inoculated pigs at various times post-inoculation

<table>
<thead>
<tr>
<th>Pigs (n)</th>
<th>Time p.i.</th>
<th>Diarrhoea at euthanasia*</th>
<th>Enlarged MLN</th>
<th>Severity of villus atrophy</th>
<th>Rotaviral antigens†</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>13 h</td>
<td>Yes</td>
<td>No</td>
<td>None</td>
<td>- + - + - + - -</td>
</tr>
<tr>
<td>4</td>
<td>24 h</td>
<td>Yes</td>
<td>Slight</td>
<td>Mild</td>
<td>+ + - - + + + +</td>
</tr>
<tr>
<td>4</td>
<td>48 h</td>
<td>Yes</td>
<td>Yes</td>
<td>Moderate to widespread</td>
<td>- - + - - - + +</td>
</tr>
<tr>
<td>2</td>
<td>72 h</td>
<td>Yes</td>
<td>Yes</td>
<td>Moderate to widespread</td>
<td>+ + - + + + +</td>
</tr>
<tr>
<td>2</td>
<td>96 h</td>
<td>Yes</td>
<td>Yes</td>
<td>Moderate</td>
<td>+ - - - + +</td>
</tr>
<tr>
<td>2</td>
<td>7 days</td>
<td>No</td>
<td>Yes</td>
<td>None</td>
<td>- - - - - -</td>
</tr>
</tbody>
</table>

* Diarrhoea present if rectal faecal consistency score ≥ 2.
† Viral antigen was either present (+) or absent (−) as determined by direct FA on small intestinal impression smears. Each symbol (+ or −) represents a pig.

The greatest number of antigen-positive cells were in the epithelial cells of jejunal and ileal villi at 24 h p.i. (Fig. 3). The greatest cellular fluorescence intensity (evaluated subjectively) was observed in the intestinal epithelial cells from 13 to 96 h p.i.

Morphological changes in tissues following inoculation with Wa HRV

As seen with distribution of viral antigens, the most significant macroscopic and microscopic changes were restricted to the gastrointestinal tract and MLN of the virulent Wa HRV-inoculated pigs. Macroscopically, all pigs had curdled milk in the stomach and variable amounts of chyle within mesenteric lymphatics at euthanasia. However, from 24–72 h p.i., the mesenteric lymphatics of the virulent Wa HRV-inoculated pigs contained less chyle than either the attenuated Wa HRV-inoculated pigs or controls. The small intestines of the virulent Wa HRV-inoculated pigs were distended with abundant opaque white-to-pale yellow fluid and had thin walls from 48–72 h p.i. The MLN of the virulent Wa HRV-inoculated pigs contained less chyle than either the attenuated Wa HRV-inoculated pigs or controls. The small intestines of the virulent Wa HRV-inoculated pigs were distended with abundant opaque white-to-pale yellow fluid and had thin walls from 48–72 h p.i. The MLN of the virulent Wa HRV-inoculated pigs were distended compared to controls at 48–96 h p.i. By 7 days p.i., no gross differences were observed in the small intestines of the Wa HRV-inoculated and control Gn pigs although the spiral colons of the virulent Wa HRV-inoculated pigs were small (half the size of controls) and their MLN remained enlarged (twice the size of controls). Significant gross changes were not seen in the attenuated Wa HRV-inoculated pigs except for mild enlargement of their MLN at 72 h p.i.

Photomicrographs depicting the typical microscopic lesions observed in the virulent Wa HRV-inoculated pigs at 13, 24 and 72 h p.i. and 7 days p.i. are shown (Fig. 4a–e). Loss of normal mature absorptive cells, the detachment of absorptive cells from their basement membranes (especially over villi tips) and mild lymphoreticular hyperplasia within villus tip stroma were seen in the duodenum and jejunum in 2 of 2 pigs at 13 (Fig. 4a, b). No significant lesions were seen in the ileum of pigs at 13 h p.i. By 24 h p.i. (Fig. 4c), loss of absorptive cell vacuolation, absorptive cell detachment and lymphoreticular hyperplasia was evident throughout the small intestine and areas of significant villous atrophy were seen. At least one region of small intestine in each pig (n = 4) had significant villous atrophy at 24 h p.i. while multiple regions had significant villous atrophy at 48 and 72 h p.i. At 96 h p.i., only the jejunum and ileum had lesions of villous atrophy. Crypt hyperplasia was seen in association with villous atrophy from 48–96 h p.i. Hyperplasia of organized gut-associated lymphoid tissues [including Peyer's patches (PP) and MLN] was seen at 24 h p.i. but was most prominent at 72 and 96 h p.i. (Fig. 4d) and persisted to day 7 p.i. (Fig. 4e). A distinct transient increase in the number of small intestinal intra-epithelial lymphocytes was also observed at 72 and 96 h p.i. At day 7 p.i. (Fig. 4e) villus morphology was within normal histological limits.

Although significant villous atrophy was not observed in any of the intestinal sections from the attenuated Wa HRV-inoculated pigs, minor microscopic changes were evident within the villous epithelial cells at 13 and 24 h p.i. Scattered duodenal and jejunal villous absorptive cells had lost normal cytoplasmic vacuolation as was observed in intestinal villous epithelial cells from virulent Wa HRV-inoculated pigs at this same time. However, affected epithelial cells remained attached to their respective basement membrane and normal cellular morphology returned by 48 h p.i. No further morphological
changes or lesions were observed in tissues from the attenuated Wa HRV-inoculated pigs except for the presence of mild lymphoid hyperplasia in the PP and MLN at 72–96 h p.i. and 7 days p.i. The relationship between the onset of diarrhoea and the distribution of viral antigens and morphological lesions in the virulent Wa HRV-inoculated pigs is depicted (Table 3). The presence of virus antigen (as detected by FA) but not villous atrophy correlated with the onset of diarrhoea.

Discussion

In this study, Gn pigs inoculated with a heterologous virulent rotavirus (virulent Wa HRV) developed clinical signs and lesions characteristic of homologous virulent rotavirus infections in children (Bishop et al., 1973; Barnes & Townley, 1973; Davidson & Barnes, 1979; Korting & Fiehring, 1983), pigs (Collins et al., 1989; Theil et al., 1978; McAdaragh et al., 1980), lambs (Snodgrass et al., 1977), calves (Hall et al., 1993; Castrucci et al., 1983) and mice (Adams & Kraft, 1967; Pappenheimer & Enders, 1947). The occurrence of diarrhoea and morphological lesions following HRV infection has been reported before in heterologous hosts, but only in Gn calves (Mebus et al., 1977) and neonatal mice (Gouvea et al., 1986). The Gn pig infected with virulent Wa HRV, however, differs from other animal models of heterologous rotavirus infection in that development of disease (and lesions) appears to be independent of the inoculation dose. For example, high doses (> 10⁵ TCID₅₀) of the MET strain (G3 serotype) of HRV elicited disease and histological lesions in neonatal mice whereas no disease or lesions developed at lower doses (Gouvea et al., 1986). By comparison, sections of intestine from a single Gn pig inoculated with a low dose (~ 10⁵ f.f.u.) of virulent Wa HRV collected at 48 h p.i. showed widespread jejunal and ileal villous atrophy microscopically (L. A. Ward & L. J. Saif, unpublished) and Gn pigs inoculated with virulent Wa HRV develop diarrhoea regardless of dose (Table 1). Whether multiple passages of Wa HRV in Gn pigs influenced the pig virulence of our virulent HRV strain is unclear; however, the outcome of virulent Wa HRV infection in Gn pigs appears analogous to infection with virulent rotavirus of homologous rather than heterologous species origin (Graham et al., 1984; Snodgrass et al., 1977; Tzipori et al., 1989; Hall et al., 1993).

Duodenal biopsies from humans diagnosed with rotavirus gastroenteritis do not always show morphological lesions (Fiehring et al., 1984; Kohler et al., 1990). In the present study of HRV infection and disease, only 58% of the virulent Wa HRV-inoculated pigs developed lesions of duodenal villous atrophy and these lesions were found only between 24–72 h p.i. and were sparsely distributed. Thus, acknowledging that many factors related to the virus strain, host origin, inoculating dose and age or susceptibility of the host probably influence the development of disease and lesion distribution, our findings suggest that the absence of lesions in duodenal biopsies may simply be an artifact of sampling error or ‘missed’ lesions.

The presence of viral antigens within cells is suggestive of active virus replication, or in the case of lymphoid tissues such as MLN, may represent uptake and processing of antigen by resident macrophages or antigen-presenting cells. In the virulent Wa HRV-inoculated pigs, the greatest number of virus-containing cells were found in the lower small intestine (jejunum and ileum). Such a distribution correlates well with the preferential replication of rotaviruses in differentiated villous epithelial cells (Saif, 1990b; Greenberg et al., 1994). In addition, the duration of detectable intestinal cellular fluorescence paralleled that of virus shedding, further suggesting that the intestinal cellular fluorescence reflects active viral replication within these cells. On the other hand, the MLN cellular fluorescence is more likely a sequela of extensive virus antigen uptake by resident phagocytic cells and/or antigen presenting cells at the peak of viral replication in the draining site (intestine). This is supported by the finding of few fluorescing cells within the MLN (< 1%) that were of a markedly reduced fluorescence intensity compared to intestinal cells and by the detection of the intracellular antigen at 24 h p.i. only, the same time at which the greatest number of intestinal cells contained viral antigen (Table 2) and at the peak of viral replication as determined by faecal viral titres. Additional studies such as examination of thin sections by EM or in situ hybridization are needed to resolve the significance of the MLN cellular fluorescence. Unfortunately, such studies may prove difficult if not inconclusive in view of the extremely low percentage of MLN cells containing virus antigen.

The inability to detect viral antigens in any of the tissues following inoculation with attenuated Wa HRV suggests that either no infection took place or that too few cells were infected or virus replication was so limited that it was not detectable by FA. In the infectivity study, the serum antibody levels of those pigs that received attenuated Wa HRV and seroconverted by 21 days p.i. were similar to those of the virulent Wa HRV-inoculated pigs at 21 days p.i. regardless of the inoculating dose of virus and all pigs (100%) receiving ≥ 10⁵ f.f.u. of attenuated Wa HRV seroconverted by 21 days p.i. By comparison, inoculation of a single pig with 2 × 10⁷ f.f.u. of inactivated attenuated Wa HRV (titre determined prior to inactivation) induced neither serum IFA nor VN serum antibodies by 21 days p.i. In addition, separate studies of protective immunity to Wa HRV in Gn pigs (Ward et al., 1996; Yuan et al., 1996), a small percentage (6%) of Gn pigs (n = 51) inoculated orally with ~ 2 × 10⁷ f.f.u. of live attenuated Wa HRV were found to shed virus and all (100%) developed detectable HRV-specific humoral and cellular immune responses by 21 days p.i. These findings in conjunction with the loss of normal absorptive cell morphology in the duodenum and jejunum of attenuated Wa HRV-inoculated but not sham-inoculated pigs at 13–24 h p.i. suggest that attenuated Wa
HRV probably infects and replicates at doses $\geq 10^7$ f.f.u. to a limited extent in young Gn pigs.

The development of villous atrophy correlated with the peak infectivity titres and the greatest number of double-shelled virus particles in intestinal contents (24–48 h p.i.). Rapid cell lysis and destruction at the peak of virus replication with release of virus particles before maturation to triple-layered rotavirus particles is completed would account for the presence of numerous empty and double-layered virus particles at 24 h p.i. The peak titre of virus shed per ml of faeces exceeded the inoculation dose by at least 2 logs which is further indicative of extensive (or efficient) virulent Wa HRV replication within the heterologous Gn piglet host.

As suggested for rotavirus-induced diarrhoea in children (Stintzing et al., 1986), Wa HRV-induced diarrhoea in Gn pigs is probably induced in part by villous atrophy and subsequent malabsorption. However, diarrhoea preceded the development of villous atrophy following virulent Wa HRV inoculation into Gn pigs, suggesting that other as yet unidentified factors probably contribute to Wa HRV-induced disease expression in Gn pigs. The development of diarrhoea prior to villous atrophy has been reported with homologous virulent rotavirus infections in pigs, calves and mice (Collins et al., 1989; Theil et al., 1978; McAdaragh et al., 1980; Mebus, 1976; Pappenheimer & Enders, 1947). Recent studies of other enteric diseases in which lesions of villous atrophy and lymphoid hyperplasia predominate suggest that host factors (such as activated T cells and cytokines) contribute to disease expression and lesion development (MacDonald & Spencer, 1988; Breese & Enders, 1947). Viral factors may also contribute to disease expression: in vitro (Tian et al., 1995) and in vivo studies using purified SA11 rotavirus NSP4 protein (Ball et al., 1995) or high doses of an inactivated (virulent strain) rotavirus (Shaw et al., 1995) suggest that early rotavirus disease may be mediated through a viral toxin. The presence of a biologically active viral toxin in virulent but not attenuated rotaviruses could explain the dose-dependence of disease and lesion expression following heterologous ‘virulent’ rotavirus inoculation in some animals (especially if the virus displays strong host-restrictive properties) and the absence of disease and lesion expression following inoculation with any dose of heterologous ‘attenuated’ rotaviruses in animals as was observed with the attenuated Wa HRV in this study.

Several mechanisms have been postulated to explain the lack of disease following inoculation with attenuated rotaviruses. Tzipori et al. (1989) postulated that the failure of a cell culture-passaged attenuated porcine rotavirus to cause disease in Gn pigs was because the number of enterocytes infected with the virus was low and therefore only minimal mucosal damage occurred. Our studies of attenuated Wa HRV in Gn pigs concur with this hypothesis in that lesion development and disease was restricted to the virulent Wa HRV-inoculated pigs. Cell culture adaptation and propagation of Wa HRV could also lead to a virus that is less stable and less able to survive passage through the stomach; subsequently, only a few viable virus particles are available to infect cells after oral inoculation (Bass et al., 1992). Attenuation through passage in cell culture may select for a virus which has increased dependence on enzymatic activation such that it cannot infect as readily as its virulent counterpart within the intestinal microenvironment (Steel & Torres-Medina, 1984). Finally, attenuation may have led to the loss of the biological activity of certain gene products involved in disease expression (such as the product of gene 10 or the NSP4 protein) (Hoshino et al., 1995).

Clearly, more studies are needed to elucidate the mechanisms that account for the differences in virulence and pathogenicity observed between the Wa HRV strains used in this study. However, the absence of disease in the presence of gut-associated lymphoid hyperplasia and VN serum antibodies in a susceptible host species after a single dose of the attenuated Wa HRV indicates promise for the use of such strains in future vaccination strategies. The immunizing potential of attenuated HRV strains in humans will likely depend in part on the quantity and antigenic integrity of virus necessary to stimulate the gut-associated lymphoid tissue and to elicit protective immune responses. Under such circumstances, the need for stabilization (i.e. enteric-coating of the virus or micro-encapsulation) and multiple doses of virus may be essential to ensure success. Further study is required to ascertain the significance of these findings with respect to other HRV strains and the development of protective immunity to HRV.

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