A comparison of the effects of oral inoculation with Rotashield and pentavalent reassortant rotavirus vaccine (WC3-PV) on suckling CB17 scid mice

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The effects of oral inoculation into infant CB17 scid mice of two reassortant rotavirus vaccines were compared. The vaccines were Rotashield and WC3-PV, a mixture of five reassortants (G1, G2, G3, G4 and P1; pentavalent reassortant vaccine). Control mice were inoculated with a placebo. At 6 days post-inoculation (p.i.), 8 of 13 (62 %; \( P < 0.005 \) Rotashield-inoculated mice developed hepatitis and/or bile-duct obstruction compared with none of 11 mice given WC3-PV and none of 14 given placebo. In the Rotashield-inoculated mice, only serotype G3 rhesus rotavirus (RRV) was isolated from multiple sites, including intestine, liver, pancreas, spleen, blood and mesenteric lymph nodes. Recovery of RRV from Rotashield-inoculated mice followed a biphasic pattern. The two peaks of RRV recovery appeared to coincide firstly with replication in the intestine during days 1–3 p.i., and secondly with virus infection of the liver from days 10 to 15 p.i. WC3 reassortants of four different serotypes were detected only at day 1 p.i. in the intestine, liver, pancreas and blood cells from three WC3-PV-inoculated mouse pups. However, WC3-PV did not produce any hepatopathology. Rotashield and WC3-PV appeared to exhibit different biological activity in infant CB17 scid mouse pups.

INTRODUCTION

Two live multivalent reassortant rotavirus vaccines, Rotashield [rhesus rotavirus (RRV) tetravalent vaccine, containing serotypes G1–G4 (RRV-TV; Wyeth Lederle Vaccines, Philadelphia, PA, USA)] and WC3-PV (bovine strain WC3 pentavalent reassortant vaccine of serotypes G1–G4 plus P1; Merck & Co., West Point, PA, USA), have been developed for use in infants (American Academy of Pediatrics, 1998; Christy et al., 1993; Clark et al., 1996a, b). In Rotashield, serotypes G1, G2 and G4 are reassortants on an RRV background. RRV itself provides the G3 component. The infant dose is \( 4.0 \times 10^5 \) p.f.u. The vaccine was licensed in the USA in August 1998, and then withdrawn in October 1999, because of association with an excess number of cases of intussusception in vaccinated infants (Centers for Disease Control, 1999). A second vaccine, RotaTeq, currently under development, is made up of reassortants of serotypes G1–G4 and P1 on a strain WC3 (bovine rotavirus) background (Clark et al., 1996a). The infant dose is approximately 100-fold higher than the Rotashield dose.

An evaluation of biological differences between these rhesus and bovine reassortant viruses may be useful. Certain strains of rotavirus of serotype G3, particularly RRV, but also SA11 and HCR3, cause hepatitis and bile-duct obstruction in suckling mice (Petersen et al., 1997, 1998; Riepenhoff-Talty et al., 1993; Uhnoo et al., 1990). WC3 has been shown not to infect liver cells in the same mouse model (Uhnoo et al., 1990). To date, no studies have been done to determine if the hepatopathogenic potential of RRV, SA11 or HCR3 is maintained when these wild-type G3 rotaviruses are reassorted.

In the present study, we investigated whether Rotashield and WC3-PV would produce infection and disease in infant CB17 scid mice. We compared the effects of oral inoculation of these two vaccines by examining clinical, morphological, histopathological and virological parameters after inoculation.

METHODS

Animals. Rotavirus-free, mid-term pregnant CB17 scid dams (\( n = 14 \)) were obtained commercially (Harlan Sprague–Dawley, Indianapolis, IN, USA). The reasons we chose this animal model are indicated in the Discussion. For the series of experiments performed, 56 pups
were sacrificed. Dams and their litters were randomly assigned to one of three experimental groups: (i) Compound H (Rotashield); (ii) Compound I (control, medium only); and (iii) Compound M (WC3-PV). Investigators were blind to the identity of the three different inocula. Mice were maintained under rigidly controlled conditions to minimize the risk of cross-contamination or exposure to extraneous bacterial or viral agents. Each dam and her subsequent litter were kept in an individual isolator cage equipped with a HEPA (high-efficiency particulate air) filter, and these cages were kept in a laminar-flow hood (Contamination Control). All food, water, bedding and cages were sterilized before use. Procedures involving the mice were carried out within the laminar-flow hood. The animal experiments were done in accordance with the National Institutes of Health Guide for the Care and Use of Animals and were approved by the SUNY at Buffalo Institutional Animal Care and Use Committee.

**Virus and inoculation.** Inocula were provided and coded by Merck Research Laboratories, West Pt, PA, USA (Rotashield was from vials purchased ‘over the counter’). The code was broken after completion of data analysis.

A lyophilized preparation of Rotashield contained one rhesus strain, RRV-G3, and three rhesus–human reassortant rotaviruses. Each reassortant rotavirus contained 10 gene segments from RRV and one gene (segment 9) from human rotavirus, which represented serotypes G1, G2 or G4. This preparation, RRV-TV, was reconstituted in 1 ml Eagle’s minimal essential medium (EMEM), and 1-day-old pups were inoculated orally with 20 μl containing 8 × 10⁵ p.f.u. (2 × 10⁵ p.f.u. of each strain) of virus. WC3-PV contained five bovine–human reassortants, and is identical to RotaTeq in terms of the virus reassortants included and their concentration. RotaTeq differs only in that the virus is suspended in a different buffer/stabilizing diluent. Each bovine–human reassortant rotavirus contained gene segments from bovine strain WC3 and human gene segment 4 or 9 (derived from human rotaviruses representing serotypes P1a, G1, G2, G3 or G4). Reassortants G1 and G2 also contain gene 3 from a human G1 rotavirus strain (W179) (H. F. Clark and others, unpublished results). One-day-old pups were inoculated orally with 20 μl containing 5 × 10⁶ p.f.u. WC3-PV (1 × 10⁶ p.f.u. per strain) diluted in Williams’ E medium (Hyclone) containing 12.5 μg trypsin ml⁻¹ (Gibco-BRL). Each dose represented 1/20 of the quantity of infectious virus administered in studies of human infants. One-day-old control group pups were inoculated with 20 μl of medium alone.

**Clinical disease follow-up and diagnostic criteria.** Animals were observed daily for signs of diarrhea and hepatitis. The clinical diagnostic criteria for diarrhea were watery or soft stools with mucus. The diagnostic criteria for hepatitis were established based on evidence from our previous studies (Riepenhoff-Talty et al., 1993; Uhnoo et al., 1990). Pups were diagnosed with hepatitis when they met two or more of the following criteria: (i) clinical signs including specific findings, e.g. jaundice with or without white- or clay-coloured faeces, and non-specific findings, e.g. malnourished appearance, lethargy and greasy fur; (ii) virus isolation was positive for rotavirus in the liver tissue; (iii) rotavirus antigen was detected in the liver tissue; (iv) gross liver appearance was abnormal.

**Animal dissection/specimen collection.** Pups were sacrificed according to a dissection schedule, approximately every other day post-inoculation up to 23 days. Animals exhibiting signs of disease were generally sacrificed at the onset of these signs. Pups were anaesthetized with Halothane (Halocarbon Laboratories). Cardiac blood was collected and, for pups at or less than 5 days post-inoculation (p.i.), whole blood was suspended in EMEM. For those pups sacrificed at day 7 p.i. or after, blood was centrifuged, serum and cell pellets were separated and both were suspended in EMEM. All blood samples were kept at −70°C for further use. Gross examination of liver, bile duct, gall bladder, spleen and intestine was carried out before sampling. The liver was examined for evidence of macroscopic changes such as areas of necrosis, grey or white patches and abnormal colours (grey, yellow, dark-bile). Pups were also examined for bile-duct obstruction, defined as an enlarged gall bladder engorged with dark bile.

Tissue samples of intestine, spleen and liver were removed and distributed as follows: in 0.5 ml EMEM for virus isolation; embedded in Tissue-Tek OCT compound (Sakura Finetek) and frozen in isopentane/liquid nitrogen for immunofluorescent antibody (IFA) assay; in 0.5 ml 10% formalin for haematoxylin and eosin (H&E) histochemical staining and immunoperoxidase antibody (IPA) assay. In order to ensure consistency of sampling, the samples were taken and processed as follows: the first 0.5 cm of duodenal tissue was taken from the proximal point and placed in EMEM, the second 0.5 cm of adjacent tissue was placed in OCT and the third 0.5 cm in formalin. For the jejunal biopsy, 1 cm of the mid-portion was collected for H&E staining and IPA assay, the right 1 cm for IFA assay, and the left 1 cm for virus isolation; for the ileal biopsy, 1 cm of the ileum was taken for virus isolation from the distal point, the adjacent 1 cm for IFA assay and the next 1 cm for H&E staining and IPA assay. The ileocecal junction, colon contents and pancreas (if found) were also collected for virus isolation. The spleen was measured and cut into three equal parts for virus isolation, IFA plus IPA assays and H&E staining. Samples of liver tissue were taken as follows: the left lobe was cut into three pieces for virus isolation, IFA plus IPA assays and H&E staining; all other lobes were collected for virus isolation. All samples in OCT and EMEM were stored at −70°C until further use. All tissue samples for virus isolation were weighed before suspension in EMEM in order that virus titres could be determined on the basis of p.f.u. (g tissue)⁻¹. Samples in formalin were embedded in paraffin on the day of collection. To avoid contamination, all dissection instruments were sterilized prior to use and a different set was used for each pup. The instruments used for one pup were disinfected in ethanol between each tissue sampling.

**IPA assay.** Tissues (intestine, liver, spleen) fixed in formalin were embedded in paraffin, sectioned and subsequently assayed for rotavirus antigen. The primary antibody (rabbit anti-human rotavirus antibody) was purchased from Dako. The detection procedure using rabbit polyclonal antibody and an HRP detection system was done according to the supplier’s instructions (Signet Laboratories). Positive and negative controls were included for each assay. Sections were scored as either positive or negative when compared with the controls.

**IFA assays.** Sections of liver (5 μm) were used for IFA assay. Tissue sections were fixed in cold (4°C) acetone for 10 min. After rehydration in PBS, 10 μl of rabbit anti-rotavirus antibody (Dako) diluted 1:10 in PBS was added and the slides were incubated overnight at 4°C. After washing the slides three times in PBS, 10 μl fluorescein-conjugated swine anti-rabbit antibody (Dako) diluted 1:20 in 0.005% Evans’ blue was added to the tissue. Slides were incubated at 37°C for 30 min. After further washes, the slides were air-dried and covered with PBS/glycerol and a cover-slip. Slides were examined with a BH-2 Olympus microscope equipped with a mercury vapour bulb. Positive and negative controls were included for each assay and slides were read as positive (appearance of specific apple-green fluorescence) or negative (lack of fluorescence) compared with controls.

**Virus isolation and identification.** Tissue samples (including liver, spleen, various intestinal tissues and pancreas) were minced with scissors and then frozen in a dry-ice/ethanol bath and thawed in a 36°C water-bath. The freeze–thaw cycles were done three times. Blood-cell clots were resuspended for 1 min before being subjected to three freeze–thaw cycles. Serum was also subjected to three
freeze–thaw cycles. All samples were then vortexed for 1 min and centrifuged for 30 s at 1100 g. Undiluted supernatant fluid from all the samples was assayed.

Quantities of infectious rotavirus were titrated using a previously described plaque assay (Offit et al., 1983). To avoid bias, every individual plaque that was well separated was harvested from all wells that contained 20 plaques and resuspended in Dulbecco’s minimal essential medium containing 0.5 μg trypsin ml⁻¹ (crystalized; Sigma) with 20 U penicillin ml⁻¹ and 20 μg streptomycin ml⁻¹. Individual plaque suspensions were inoculated onto a monolayer of MA104 cells (Whittaker Bioproducts) in a 24-well plate (Falcon, Becton Dickinson). Plates were checked daily until the maximum cytopathic effect was reached. Rotavirus plaques were characterized by PAGE RNA electrophoretotyping (Dolan et al., 1985) and compared with positive controls (the inoculum viruses). Samples with a faint banding pattern were subjected to a second passage in MA-104 cells and were reanalysed. Results were considered negative if rotavirus-specific RNA was not detected by PAGE.

Statistics. Data were analysed by a χ² test. All comparisons were made between the two vaccine groups and did not include the placebo controls.

RESULTS

Fifty-six newborn CB17scid pups were inoculated orally with Rotashield, WC3-PV or placebo. After inoculation, they were observed daily and euthanized and dissected at intervals for macroscopic and microscopic examination of pathological changes. Eighteen pups were sacrificed in the group of mice receiving Rotashield, 18 in the WC3-PV group and 20 in the placebo group (Table 1). None of the mice given placebo developed any signs of disease. Two of the mice given WC3-PV developed mild diarrhoea, but no other disease signs were observed. Oral inoculation of Rotashield resulted in diarrhoea, hepatobiliary disease and, in some cases, death.

Diarrhoea

Six of 18 (P>0.05) of the mice given Rotashield developed mild (stool volume less than 10 μl) to moderate diarrhoea (stool volume 10–30 μl) from days 2 to 4 p.i. Two of the pups, SH3 (which had had diarrhoea for 1 day) and SH5 (which had had diarrhoea for 2 days at the time of autopsy) (both at day 3 p.i.), had RRV isolated from their intestinal tissues and colon contents. Two of 18 of the mice given WC3-PV had mild diarrhoea on day 1 p.i. The stool volumes of these two mice were too small to allow collection and the mice were not sacrificed on day 1 p.i., but were monitored for additional signs of disease and sacrificed later. There was no evidence of any clinical disease after day 1 p.i. in any WC3-PV-inoculated mice. However, as discussed later, virus was isolated from three WC3-PV-inoculated mice that were sacrificed on day 1 p.i. None of the mice given placebo developed any signs of diarrhoea.

Hepatitis and bile-duct obstruction

A total of nine mice that received Rotashield had evidence of developing hepatitis (Table 2). However, since data from our previous (Uhnno et al., 1990) and present studies have shown an incubation period of approximately 7 days for development of hepatitis or bile-duct obstruction after oral inoculation of RRV, we have chosen as our denominator the number of mice remaining alive (not sacrificed or dead) after day 6 p.i. For any mice sacrificed from days 1 to 6 p.i., the incidence of hepatitis was 62% (8/13) (P<0.005), compared with none of 11 mice given WC3-PV.

The hepatic disease varied from asymptomatic to fatal. Clinical signs, especially jaundice and leshargy, appeared in mice as early as day 5 p.i. and as late as day 16 p.i. The mean number of days p.i. when the first signs of hepatitis were seen was 10.3. The first case of hepatitis was identified 3 days p.i. The affected mouse, SH3, exhibited no clinical signs of hepatitis, but was diagnosed by the recovery of infectious virus by plaque assay and by detection of rotavirus antigen in the liver by both IPA and IFA assays. The second pup (no dissection code) diagnosed with hepatitis was found jaundiced and lethargic on day 6 p.i. and was found dead on the following day. Tissue samples

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>No. of pups sacrificed</th>
<th>No. of pups dissected at each time period (days p.i.)*</th>
<th>No. of pancreas glands collected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1–6</td>
<td>7–14</td>
</tr>
<tr>
<td>Rotashield</td>
<td>18</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>WC3-PV</td>
<td>18</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Placebo</td>
<td>20</td>
<td>6</td>
<td>9</td>
</tr>
</tbody>
</table>

*Days 1–6 p.i., 1, 3, 5; days 7–14 p.i., 7, 9, 10, 11, 13; days 15–23 p.i., 15, 17, 21, 23.
were not collected from this pup. Pups SH7 and SH11 had mild jaundice and lethargy for 2 days, days 5 and 6 p.i., and autopsy revealed gross abnormalities in the liver. Hepatitis in pup SH23 was diagnosed by positive clinical signs and the recovery of infectious virus from the liver. Pup S4H15 had lethargy with a normal gross appearance of the liver, but an enlarged gall bladder, and virus was isolated from and viral antigen detected in the liver. Three other pups, S4H10, S5H13 and SH15, had severe clinical disease, observed as jaundice, lethargy, white faeces and greasy fur. The gross morphological changes in the livers of these three pups included a grey-, yellow- or bile-coloured appearance. Microscopic examination of H&E-stained sections from the liver of pup SH15 revealed multi-focal, non-zonal necrosis (about 5%) associated with mononuclear infiltration; scattered neutrophils were also present in the liver. The recovery of infectious virus and the detection of rotavirus antigen in the livers of all three mice confirmed the diagnosis of hepatitis. In addition to hepatitis, three pups (S4H10, S5H13 and S4H15) also appeared to develop bile-duct obstruction, suggested by an enlarged gall bladder engorged with dark bile. In pup S4H15, the liver was grossly and microscopically normal in the presence of the enlarged gall bladder.

Hepatobiliary disease associated with Rotashield was not confined to particular litters, because evidence of infection could be found in pups in every cage. However, no cage contained pups that were all equally affected. Further evidence that disease and death were randomly distributed among litters was the finding that three missing pups among the Rotashield-inoculated mice, presumed to have been cannibalized by their mothers after the onset of disease, were each members of different litters. This exclusive association of Rotashield with disease was reinforced by the appearance of disease only in cages containing Rotashield-infected animals.

Mice that received WC3-PV or placebo did not show any signs of developing hepatitis. Although infectious virus was isolated from the livers of mice inoculated with

Table 2. Clinical and laboratory findings in mice inoculated with Rotashield

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Day p.i. sacrificed</th>
<th>Clinical signs and onset time</th>
<th>Gross organ description</th>
<th>IFA/IPA assays (liver)*</th>
<th>Virus isolation (liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH3</td>
<td>3</td>
<td>None</td>
<td>Slightly pale liver</td>
<td>RV Ag+</td>
<td>+</td>
</tr>
<tr>
<td>Not dissected</td>
<td>7</td>
<td>Jaundice, lethargy day 6 p.i.; died day 7 p.i.</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SH7</td>
<td>7</td>
<td>Mild jaundice and lethargy days 5–6 p.i.</td>
<td>Grey–white liver</td>
<td>RV Ag−</td>
<td>−</td>
</tr>
<tr>
<td>S4H10</td>
<td>10</td>
<td>Jaundice, lethargy and malnourished day 10 p.i.</td>
<td>Yellow liver, enlarged gall bladder</td>
<td>RV Ag+</td>
<td>+</td>
</tr>
<tr>
<td>SH11</td>
<td>11</td>
<td>Mild jaundice and lethargy days 5–6 p.i.</td>
<td>Grey liver</td>
<td>RV Ag−</td>
<td>−</td>
</tr>
<tr>
<td>S5H13</td>
<td>13</td>
<td>Jaundice, white faeces day 13 p.i.</td>
<td>Dark-bile coloured liver, enlarged gall bladder and spleen</td>
<td>RV Ag+</td>
<td>+</td>
</tr>
<tr>
<td>S4H15</td>
<td>15</td>
<td>Lethargy day 15 p.i.</td>
<td>Enlarged gall bladder, normal liver</td>
<td>RV Ag+</td>
<td>+</td>
</tr>
<tr>
<td>SH15</td>
<td>15</td>
<td>Jaundice and white faeces, lethargy and greasy fur days 11–15 p.i.</td>
<td>Pale liver with scattered areas of necrosis</td>
<td>RV Ag+</td>
<td>+</td>
</tr>
<tr>
<td>SH23</td>
<td>23</td>
<td>Mild jaundice, white–yellow faeces, greasy fur days 14–16 p.i.</td>
<td>Normal liver</td>
<td>RV Ag−</td>
<td>+</td>
</tr>
</tbody>
</table>

NA, Not applicable.
*RV Ag, rotavirus antigen.

Table 3. WC3 reassortants recovered from mice inoculated with WC3-PV

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Day p.i.</th>
<th>Virus (p.f.u. g⁻¹) recovered from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Duodenum</td>
</tr>
<tr>
<td>SM1</td>
<td>1</td>
<td>6·1 × 10⁴</td>
</tr>
<tr>
<td>SM1b</td>
<td>1</td>
<td>−</td>
</tr>
<tr>
<td>SM1c</td>
<td>1</td>
<td>−</td>
</tr>
</tbody>
</table>

NC, Not collected.
*−, Failure to recover virus.
WC3-PV on day 1 p.i. (Table 3), it was not recovered at any later time, and no further evidence in terms of clinical signs, antigen detection, gross organ findings or hepato-pathological changes were seen that supported the diagnosis of hepatitis in these mice.

Detection of rotavirus antigen

As mentioned above, rotavirus antigen was detected by IPA and IFA assays in livers from five (SH3, S4H10, S5H13, S4H15 and SH15) of eight Rotashield-infected mice with hepatitis. Antigen was present in the portal areas around veins and in the parenchyma, both in single cells and in small foci of cells. An example of rotavirus antigen detected by IPA assay can be seen in Fig. 1. Intestine and spleen tissue tested by using this assay were all negative for rotavirus antigen. The mesenteric lymph nodes (MLN) and samples from the pancreas were too small for immunohistochemical assays. Only virus isolation was attempted with these samples. No virus was detected in any tissue sample from mice that received WC3-PV or placebo.

Virus isolation

Rotavirus was isolated from only three WC3-PV-inoculated mice (SM1, SM1b and SM1c) on day 1 p.i., as shown in Table 3. Low titres of virus ($10^2$–$10^4$ p.f.u. g$^{-1}$) were isolated from many organs, including the livers of all three mice. Three different reassortants, WC3-G1, G3 and G4, were identified.

Recovery of virus from Rotashield-inoculated mice followed a biphasic pattern (Table 4). Each of three mice sacrificed from days 1 to 3 p.i. exhibited low-titre infections, primarily in the gut. From 5 to 9 days p.i., virus was recovered from the intestine of only one of five animals and not from any other tissues. A resurgence of virus was detected throughout the viscera from days 10 to 15 p.i. Virus was recovered from four of five mice during this time period. Particularly high titres ($>10^7$ p.f.u. g$^{-1}$) were identified in the liver and pancreas. After day 15 p.i., the rate of infection declined again. In five surviving animals, virus was recovered only from the MLN ($10^5$ p.f.u. g$^{-1}$) of pup S4H21 and the liver ($2.4 \times 10^4$ p.f.u. g$^{-1}$) of pup SH23.

Identification of virus recovered from tissues of Rotashield-infected mice was carried out by PAGE RNA electrophorotyping. One to 20 plaques were examined from each positive tissue (Table 5): 368 plaques were identified as RRV-G3. No reassortants were recovered.

DISCUSSION

We found biological differences with respect to the replication, invasiveness and pathogenic potential among viruses in Rotashield and viruses in WC3-PV following their inoculation into CB17$^{\text{scid}}$ mice. Mice inoculated with WC3-PV demonstrated no signs other than very mild diarrhoea, although the WC3-PV viruses produced a viraemia on day 1 p.i. Mice inoculated with Rotashield developed diarrhoea, hepatitis and bile-duct obstruction, and there were some deaths. This is the first report demonstrating that RRV can retain its hepatopathogenic potential in mice when administered in the presence of human–RRV reassortants of equal titre. We also observed two distinct peaks of rotavirus recovery from inoculated mice, correlating with RRV replication in the intestine and subsequently in the liver. High titres of RRV were also recovered from the pancreas.

Only RRV-G3 was isolated from samples taken from pups inoculated with Rotashield, despite the fact that the RRV-G1, RRV-G2 and RRV-G4 reassortants were present in equal proportions to RRV-G3 in the vaccine. In a study of oral inoculation of Rotashield into adult BALB/c mice, RRV-G3 was the virus most frequently isolated from samples of Peyer’s patches (PP), and predominantly RRV-G3 but also G1 and G2 reassortants were recovered from MLN (Moser et al., 2001).

In separate studies (unpublished), immunocompetent newborn BALB/c mice and gnotobiotic newborn piglets
Table 4. RRV-G3 recovered from multiple sites of mice inoculated with Rotashield

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Day p.i.</th>
<th>Virus (p.f.u. g⁻¹) recovered from:</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Ileal-caecal Colon contents</th>
<th>Spleen</th>
<th>MLN</th>
<th>Liver</th>
<th>Pancreas</th>
<th>Whole blood</th>
<th>Blood cells</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH1</td>
<td>1</td>
<td></td>
<td>8·3 x 10⁴</td>
<td>1·0 x 10⁴</td>
<td>1·0 x 10³</td>
<td>7·5 x 10⁴</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>SH3</td>
<td>3</td>
<td></td>
<td>8·3 x 10⁴</td>
<td>1·0 x 10⁴</td>
<td>1·0 x 10³</td>
<td>7·5 x 10⁴</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>S5H3</td>
<td>3</td>
<td></td>
<td>8·3 x 10⁴</td>
<td>1·0 x 10⁴</td>
<td>1·0 x 10³</td>
<td>7·5 x 10⁴</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>SH5</td>
<td>5</td>
<td></td>
<td>8·3 x 10⁴</td>
<td>1·0 x 10⁴</td>
<td>1·0 x 10³</td>
<td>7·5 x 10⁴</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>S5H9</td>
<td>9</td>
<td></td>
<td>1·0 x 10⁵</td>
<td>4·2 x 10⁵</td>
<td>1·0 x 10³</td>
<td>7·5 x 10⁴</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>S4H10</td>
<td>10</td>
<td></td>
<td>1·0 x 10⁵</td>
<td>4·2 x 10⁵</td>
<td>1·0 x 10³</td>
<td>7·5 x 10⁴</td>
<td>NC</td>
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<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>S4H11</td>
<td>11</td>
<td></td>
<td>1·0 x 10⁵</td>
<td>4·2 x 10⁵</td>
<td>1·0 x 10³</td>
<td>7·5 x 10⁴</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>S5H13</td>
<td>13</td>
<td></td>
<td>1·0 x 10⁵</td>
<td>4·2 x 10⁵</td>
<td>1·0 x 10³</td>
<td>7·5 x 10⁴</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>S4H15</td>
<td>15</td>
<td></td>
<td>1·0 x 10⁵</td>
<td>4·2 x 10⁵</td>
<td>1·0 x 10³</td>
<td>7·5 x 10⁴</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
</tbody>
</table>

NC: Not collected.

*Failure to recover virus.

were inoculated with comparable doses of Rotashield (on the basis of p.f.u. g⁻¹). In newborn BALB/c mice, virus was rarely recovered from gut, liver and MLN, and was exclusively RRV-G3. In contrast, virus recovered from visceral organs of piglets included RRV-G3, and G2 and G4 reassortants of RRV (L. Saif, M. Riepenhoff-Talty, H. Qiao & H. F. Clark, unpublished data). In clinical trials of Rotashield in infants, RRV predominated but several ST3 (rotavirus strain St Thomas 3) x RRV reassortants (G4) were also recognized (Hoshino et al., 2003).

Since various reassortants of RRV have been recovered from orally inoculated infants, piglets and adult BALB/c mice given Rotashield, but only RRV was recovered from immunocompetent (see above paragraph) or SCID mice fed Rotashield, it appears that absolute selective replication of the RRV component of animals fed Rotashield is unique to the newborn mice. As VP7 is the only protein that varies in Rotashield components, it appears that VP7

Table 5. Number of plaques recovered for each Rotashield-infected mouse and site of isolation

All plaques were RRV-G3.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of plaques for mouse:</th>
<th>Total no. of plaques</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SH1</td>
<td>SH3</td>
</tr>
<tr>
<td></td>
<td>1 d.p.i.</td>
<td>3 d.p.i.</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>Visceral (lymphocytic)</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Blood cells</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Serum</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Lymphocytic tissue is spleen or MLN. Therefore, visceral or lymphocytic tissue includes liver, pancreas, spleen and MLN.
is critical in controlling invasion of and replication within liver cells. Alternatively, since only RRV was found in the gut, RRV VP7 may be necessary only to allow replication in the gut and that it is enteric replication that might lead to viraemia. If so, the ability of the RRV VP7 reassortant to replicate in visceral organs could be determined only by parenteral inoculation. If it were determined that parenteral inoculation of Rotashield into newborn mice led to replication of Rotashield reassortant viruses in visceral organs, the observation would be of questionable significance since all natural exposure to rotavirus (as well as administration of rotavirus vaccine) is by oral inoculation. The observation that a selected heterologous host–rotavirus system involving a virus heterologous to that host causes disease (hepatitis and biliary obstruction) not demonstrated in any known natural homologous host–rotavirus system is of great interest.

The suggested critical role of VP7 in the RRV–newborn BALB/c mouse system supports previous observations that, in studies with rotavirus reassortants in pigs, genes 3, 4, 9 and 10 all influence virulence (Hoshino et al., 1995). However, in other studies (Mossel & Ramig, 2002) on orally inoculated newborn mice, gene 7 (product NSP3) was the predominant determinant of spread of rotavirus to the liver. Factors controlling hepatotropism of rotavirus in the mouse are not yet completely understood.

Although the time of appearance and pattern varied, rotavirus was detected in the bloodstream of mice after oral inoculation with both vaccines. Possible routes for transit of virus to the bloodstream are outlined in Fig. 2(a) (RRV) and (b) (WC3-PV). We suggest that virus may reach the bloodstream and viscera by two common routes: directly into the blood circulation from the intestine or indirectly through the M cells, PP, MLN and spleen. This second route was established by Dhara et al. (1988) using murine rotavirus and immunocompetent infant BALB/c mice. This pathway would play a diminished role in immunodeficient mice, although uptake by macrophages and interaction with non-specific immune cells would occur. In the current study we did not detect RRV in the bloodstream until day 10 p.i. and this appearance coincided with replication in the liver. It is likely that the small inoculum of RRV and the initiation of the resultant infection in the intestine could account for insufficient inoculum virus reaching the blood circulation. To explain how virus reaches the liver we postulate a third pathway, an ascending route through the bile ducts and hepatic ducts to the liver. There are several lines of evidence that point to an ascending route. First, as mentioned above, the lack of detectable virus circulating in the blood would be a factor. There was also the bimodal nature of virus shedding in the gut, with a distinct lag before liver infection was established. If large amounts of virus were available to the liver cells in the first 24–48 h via the blood circulation, one would not expect it to take 7–10 days for progeny virus to be detected in the liver. Furthermore, bile-duct obstruction appeared to occur at the same time or perhaps earlier than hepatitis. In addition, a previous study that documented the location of the obstruction in the RRV-infected mice showed a consistent pattern, with the lesion at the distal end of the common bile-duct where it empties into the duodenum (Riepenhoff-Talty et al., 1993).
Since WC3-PV (Fig. 2b) appeared in the bloodstream, but only within the first 24 h, it is likely that the WC3-PV viruses isolated from the blood were inoculum viruses. Additional inoculum virus was probably taken up into enterocytes, but there is no evidence that a productive intestinal infection ensued. Similarly, the brief WC3-PV viraemia would expose all visceral organs, but there were no signs of extraintestinal infection. A recent report of rotavirus antigenaemia associated with rotavirus diarrhoea in human infants and inoculated laboratory animals, primarily mice, also suggests that rotaviruses can, and do, escape the intestine (Blutt et al., 2003). The majority of infections described were homologous productive infections, and undoubtedly large amounts of rotaviral antigens were present in the intestines.

We documented the active replication of RRV in hepatocytes by positive findings with IFA and IPA assays. These assays detect intracellular (intracytoplasmic) viral antigen in fixed cells. Virus isolation is more sensitive, and detects fully infectious virions, but does not reveal the location of the virus. It does not distinguish between virus in the blood circulation of an organ or virus replicating in the cells of the organ. However, detection of large deposits of viral antigen in organ tissue is consistent with active virus replication in that organ.

The detection of relatively high titres of RRV in the pancreas of Rotashield-inoculated mice is also intriguing. However, because individual pancreatic samples were insufficient for secondary detection by antigen assay, we could not confirm RRV replication in the pancreas. Three of the four mice with high titres of RRV in the pancreas also had RRV in the blood, but in much lower titres. Follow-up studies are needed to determine whether our observation that mouse pancreas may support RRV replication is real. There is a recent report describing the growth of rotavirus in primary human pancreatic cell culture (Coulson et al., 2002).

Our findings of a low incidence of diarrhoea in the Rotashield-inoculated mice were not totally unexpected. Uhnoo et al. (1990) reported diarrhoea in 80% of CB17 scid pups inoculated with a similar dose (10^3 f.f.u.) of purified RRV. In the present study, it appears that the ability of RRV-G3 to produce diarrhoea is diminished in Rotashield, possibly by interference mediated by RRV reassortants.

In this study, we elected to keep the Rotashield titre very low to maintain the same ratio of Rotashield titre to WC3-PV titre as used in clinical practice. Preliminary studies using BALB/c mice (the IgH congenic partner of CB17 scid mice) showed only minor changes after inoculation of the similar titre of Rotashield (unpublished data). We then examined the more susceptible CB17 scid newborn mouse model, which enabled us to maintain the same relative Rotashield/WC3-PV virus titre.

We found clear biological differences between Rotashield and WC3-PV in the suckling CB17 scid mouse model. These findings confirm and extend the work of Uhnoo et al. (1990), who studied the WC3 and RRV parental rotavirus strains in the same mouse model. Data from this study again suggest that RRV has unusual and sometimes severe pathogenic potential, and the ability to cross species barriers efficiently. These observations suggest that it might not be the first choice as a vaccine candidate.

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REFERENCES


