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# Propagation of Infantile Gastroenteritis virus (Orbi-Group) in Conventional and Germfree Piglets

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Infantile gastroenteritis virus (orbi-group) recovered from stools of infants with acute nonbacterial gastroenteritis was administered per os to germfree and conventional piglets. Virus was found subsequently in stools and in the mucosal epithelial cells of the small intestine of these animals. Some animals developed diarrhea. Added proof of orbivirus replication was obtained through the use of tritiated uridine injections and the recovery of labeled virus in gut contents at the time of autopsy. Serological conversion was demonstrated in infected germfree piglets.

A specific virus referred to here as infantile gastroenteritis virus (IGV), but also called orbi-rota-, reo-like, or duovirus by other workers, has now been demonstrated in many parts of the world (7). IGV has been shown to be a major cause of nonbacterial gastroenteritis in young children, especially during the colder months of the year in temperate climates (3, 9). Morphologically similar viruses have been found to cause diarrhea in infant mice and young calves. These viruses are antigenically related to one another and to IGV (11). Other animal species including swine, sheep, and simians may also possess counterpart diarrhea-producing viruses (5).

Since our attempts to propagate IGV in cell culture systems and in organ cultures of human embryo small gut had failed, we sought an animal susceptible to infection. Once a satisfactory animal model was found, certain physicochemical characters of the virus could be determined. In addition, the animal model might also permit a study of the pathogenesis of viral diarrhea. Germfree and conventional piglets were used since gnotobiotic facilities were available to us at the University of Guelph and since colleagues in our own institution and at Guelph were engaged upon work with transmissible gastroenteritis virus in swine. Our choice of the pig was also influenced by the size of the animal and the fact that its gut tract was anatomically similar to that of the human.

In this paper we present evidence indicating that IGV replicates in both germfree and conventional piglets and that this virus contains ribonucleic acid genome. During the course of the work, we encountered cross-infection among the conventional piglets when these were housed in close approximation to one another and were fed from a common food stock.

## MATERIALS AND METHODS

**Piglets.** Germfree Yorkshire breed piglets were obtained from and housed in isolators at the Gnotobiotic Laboratory, Department of Clinical Studies, Ontario Veterinary College, University of Guelph. Animals were given 1 ml of virus in milk per os when aged 3 to 6 days. Diet consisted of sterile condensed cow's milk (Farmer's Wife, Cow and Gate Co., Bramelea, Ontario). On the 3rd day of life, piglets were injected intramuscularly with an iron-dextran complex (Imferon) containing 100 mg of elemental iron. Rectal swabs were taken daily to monitor for virus and for bacterial or fungal contamination.

Conventional littermate piglets also of the Yorkshire breed were purchased from Maple Leaf Research Laboratories, Georgetown, Ontario. They were removed from the sow when aged 2 to 3 days and transported in polystyrene cannisters in the interior of a heated car to the Research Institute, The Hospital for Sick Children, or to the Medical Sciences Building, University of Toronto. Housing arrangements for these animals are shown in Table 1. Piglets were kept in wire cages and fed Provi-milk (Provimi Ltd., Woodstock, Ontario). For additional environmental heating we used overhead infrared lamps. Animals were given 1 ml of virus in milk per os. Rectal swabs were taken twice daily.

At the times indicated in Table 1, germfree and conventional piglets were killed by intracardiac inoculation of sodium pentobarbital. Small and large bowel contents were collected by "milking" out the contents and flushing phosphate-buffered saline (PBS) through the lumen. Portions of bowel were fixed in formol saline for hematoxylin-eosin staining and in 95% ethanol for indirect immunofluorescence microscopy (IIM). After flushing exposed pieces of mucosa with PBS, small areas were scraped with a scalpel blade and deposited on microscopic slides for direct negative-contrast stain electron microscopy (EM). Samples for the above examinations were taken from the first, second, third, and fourth parts of the duodenum, the upper jejunum, mid-small bowel, and ileum.

TABLE 1. *Synopsis of seven separate experiments indicating the detection of virions and virus antigen in rectal swabs and small bowel mucosa of piglets given IGV per os*

| Exp no. | No. of littermates | No. of controls | No. given virus | Day virus 1st detected | Period of excretion (days) | Day of autopsy | Virus detected in gut tissues at autopsy | Radiolabeling |
|---|---|---|---|---|---|---|---|---|
| Germfree | | | | | | | | |
| 1 | 1 | 0 | 1 | 5[a] | 2 | 6 | ND[b] | ND |
| 2 | 8 | 1 | 7 | 4, 5, 5, 4, 4, 3, 4 | 4, 2, 2, 4, 8, 4, 4 | 8, 6, 8, 8, 18, 8, 8, 8 | 1 piglet positive 7 piglets negative | ND |
| 3 | 4 | 2 | 2 | 4, 4 | 2, 2 | 5, 5, 5, 5 | ND | Yes |
| Conventional | | | | | | | | |
| 4 | 3 | 0 | 3 | ND | ND | 4 | All 3 positive | ND |
| 5 (Both animals in same room) | 2 | 1 | 1 | Negative | Negative | 5 | Inoculated and control positive | Yes |
| 6 (Different rooms same area) | 2 | 1 | 1 | 4, 4 | 2, 2 | 5 | Inoculated and control positive | Yes |
| 7 (Separate building and personnel) | 2 | 1 | 1 | 3 | 1 | 4 | ND | Yes |

[a] Virus administered on day 1.
[b] ND, Not done.

**Virus challenge.** Piglets received 1 ml of a virus suspension per os containing approximately $10^8$ IGV particles per ml. The suspension was prepared from a stool pool of six IGV-positive patients. Virus was recovered by ultracentrifugation and resuspended in distilled water, and the suspension was assessed by negative-contrast stain EM. Commercial bovine skim milk was added to the suspension, and the material was filtered through a Sartorius membrane filter of 0.45-$\mu$m pore size. The filtrate was checked for bacterial sterility using blood agar plates.

**EM and IIM.** Small portions of stool from rectal swabs were transferred to microscopic slides and mixed with distilled water to form a suspension. A drop of this suspension was then placed on an EM grid and stained by 1.6% phosphotungstic acid at pH 6.5 (9). Mucosal scrapes on microscopic slides were treated in a similar manner.

For the detection of IGV antigen, portions of gut for IIM were processed according to Sainte-Marie (10). Sections of 4 $\mu$m in thickness were reacted and photographed as previously described (9).

**Radiolabeling.** Twenty-nine hours after virus ingestion, a series of five [$^3$H]uridine or [$^3$H]thymidine subcutaneous injections were commenced. Subsequent injections were given at 45, 53, 69, and 77 h. Infected and control piglets received 0.5 ml of [$^3$H]uridine or [$^3$H]TdR inoculum. The inoculum consisted of 3 parts stock [$^3$H]uridine (Amersham-Searle TRK 178; specific activity, 29 Ci/mmol) or 3 parts stock [$^3$H]TdR (TRK 300; 15 Ci/mmol) to 1 part sterile 4× PBS.

Approximately 96 h after ingestion of virus, piglets receiving [$^3$H]uridine or [$^3$H]TdR were killed and their bowel contents were collected. Stools were processed and virus was purified by a procedure described in a paper now submitted for publication. Briefly, the intestinal contents were agitated with glass beads and clarified by centrifugation at 5,000 × *g* in a Sorvall centrifuge for 10 min at 4 C. The supernatant was collected and centrifuged at 80,000 × *g* in an International centrifuge for 2 h at 4 C. The pellet obtained was resuspended in PBS and extracted with Freon 113 (Dupont). The aqueous phase of this extraction was again subjected to ultracentrifugation under the conditions described above. The pellet obtained was resuspended in 3 ml of PBS and made up to a density of 1.35 g/ml in optical-grade cesium chloride. This cesium chloride suspension was centrifuged at 35,000 rpm in an SB283 rotor at 4 C for 16 h. The gradient was collected into 15-drop fractions. Fractions were monitored for density of cesium chloride with a refractometer and for radioactivity with a scintillation counter.

**Antibody determination.** Antibody studies were performed on the litter of germfree piglets detailed as experiment 2 in Table 1. The antigen substrate consisted of jejunal mucosal epithelial cells from an infant who died with severe IGV. Cells were spread in wells on microscopic slides, air dried, and acetone fixed. Antibody was detected by a three-stage IIM using sera from piglets diluted 1:20 and 1:40 in stage one; anti-porcine serum (GIBCO) made in rabbit and diluted 1:5 in stage two; and for the final stage,

a goat anti-rabbit fluorescein conjugate diluted 1:5 (Hyland Laboratories). In this three-stage test system, serum incubation times were of 45-min duration. Slides after incubation with sera were washed for 30 min, using three changes of PBS.

## RESULTS

**Recovery of virus from inoculated animals.** IGV was detected by a negative contrast staining EM screening test in rectal swabs of piglets after a single ingestion of the virus. Both germfree and conventional piglets excreted virus. Virus excretion was detected as early as day 3. In the one piglet where autopsy was deferred until day 18, virus excretion lasted 8 days. The seven separate trials which we conducted are summarized in Table 1. The table also shows that virions or virus antigen could be detected in the mucosa of the small intestine by EM or IIM if an autopsy was conducted within 6 days or less of virus ingestion. Figure 1 shows virus antigen by IIM in the columnar epithelial cells of the duodenal mucosa. The distribution of antigen was limited to discrete foci in the upper small bowel. Larger and more numerous foci were found in the upper duodenum. Fewer and smaller foci of virus antigen were detected in the distal duodenum and upper jejunum.

FIG. 1. *Indirect immunofluorescence photomicrograph of piglet duodenal mucosa showing foci of columnar epithelial cells containing IGV antigen in the cytoplasm distal to the nuclei.* × 270.

At autopsy the mucosal aspect of the small bowel of infected piglets was generally congested in appearance. On histological examination, vascular congestion was also apparent. Light microscopy of stained sections also showed a patchy bluntening of villi, and the cells at the tips of villi in these areas tended to be cuboidal rather than columnar in shape.

**Clinical observations.** Actual vomiting was not witnessed by us in either germfree or conventional piglets. Diarrhea in germfree piglets unfortunately is difficult to assess since these animals usually produce very loose stools. However, in experiment 3 (Table 1) the two infected germfree animals had more liquid and frequent stools on day 4 in comparison with the two control germfree piglets. A similar observation was not made in experiment 2. Conventional piglets exhibited diarrhea beginning 32 h after virus ingestion, and this lasted for 24 h.

**Serological response.** All seven infected germfree piglets (experiment 2) at the time of autopsy showed detectable antibody at serum dilutions of 1:20. Six of these animals also had detectable antibody when sera were diluted 1:40. Umbilical cord blood from an infected piglet and from the control piglet did not contain antibody. Antibody was not detected in the control animal on day 8 of the experiment when this piglet was killed.

**Cross-infection.** Whereas none of the germfree control animals showed demonstrable virus, conventional control piglets in experiments 5 and 6 (Table 1) became secondarily infected by IGV. In experiment 5 the two piglets were housed in adjacent cages in the same room. A common food source was used, and two of the authors who tended the animals shared duties. Virus infection was again demonstrated in the control piglet in experiment 6 (Table 1) even though piglets were housed in separate rooms in the same general area. Although on this occasion different personnel tended the animals, the food source used was still common to both piglets. Diarrhea developed in the deliberately infected piglet at 32 h after virus ingestion and continued for a period of 24 h. The control animal developed diarrhea 56 h after the experiment commenced, at which time the deliberately infected piglet had ceased passing frequent liquid stools. In experiment 7 (Table 1) piglets were housed in separate buildings, attended by different personnel, and provided with separate food sources. In this last experiment, the control piglet remained free of detectable virus.

**In vivo labeling.** Four in vivo labeling experiments (see Table 1) were conducted. Three of these used tritiated uridine and conventional

piglets. In the fourth experiment performed in germfree piglets, virus-infected and control animals were either given [$^3$H]uridine or [$^3$H]TdR. When [$^3$H]uridine was injected subcutaneously for in vivo labeling and the virus recoverable in bowel contents at autopsy was purified, a peak of radioactive material was regularly found in CsCl gradient fractions corresponding to a density of 1.36 g/ml (Fig. 2). This peak fraction contained virus readily demonstrable by EM. Control animals failed to show a similar peak of radioactivity even though in experiments 5 and 6 (Table 1) these so-called "controls" were shown to be virus infected. Optical density readings of the CsCl gradient fractions at 260 nm were performed in experiment 6. Whereas purified gut contents from both the control and the deliberately infected piglet showed an optical density peak at 260 nm, the deliberately infected animal only provided a peak of radioactive material in a CsCl gradient fraction corresponding to a density of 1.36 g/ml. In experiment 3 using germfree piglets (Table 1), material from both controls failed to show a peak of radioactivity. The [$^3$H]uridine-injected, virus-infected piglet but not the [$^3$H]TdR-injected, virus-infected piglet provided bowel contents that gave a peak of radioactive material at a density of 1.36 g/ml.

## DISCUSSION

We have shown that IGV infects both conventional and germfree young piglets. The amount of virus produced by infected animals greatly exceeded the quantity of virus ingested since random sampling from approximately 100 ml of intestinal contents and washings collected at autopsy from each piglet showed detectable virus by a simple EM screening test. Virus antigen was demonstrable using IIM. Positive fluorescence was found only in the distal cytoplasm of the mucosal columnar epithelial cells and resembled the pattern seen in fatal human IGV infections (9) and in duodenal biopsy specimens of young children with acute enteritis due to IGV (4). Both the number of foci showing antigen-positive cells and the size of each focus were comparatively small in piglets when compared with our observations in fatal human

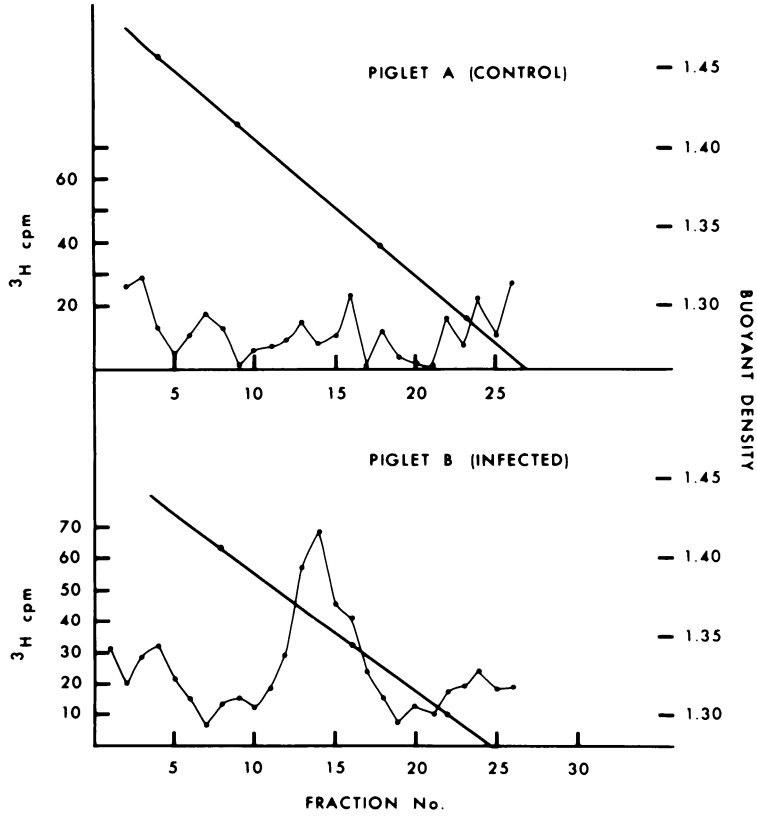


FIG. 2. *Distribution of radioactivity from stool extracts on CsCl density gradient fractions in control piglet (A) and infected piglet (B). Virus was detected by EM in the peak fractions 13, 14, and 15.*

cases. Moreover, virus antigen in piglets was only found in the upper small bowel, whereas in the fatal human cases we have studied, positive fluorescence was detectable throughout the entire small bowel including the distal ileum. The histological changes in infected piglets also resembled the histological appearances described in human IGV infections (1, 2).

Clinical evidence for disease among the infected piglets was admittedly poor. When diarrhea did occur, it lasted no longer than 24 h. Our observations of clinical disease in germfree animals was compromised by the fact that these animals normally pass a very loose stool. The relatively mild disease seen in piglets correlates with our IIM findings of comparatively small, widely separated foci of detectable virus antigen.

Germfree piglets infected with IGV developed antibody. We could not, however, convincingly detect this serological response by a conventional two-step IIM technique, or by a complement fixation test (8) using a crude human stool antigen or by our recently evaluated system of counterimmunoelectroosmophoresis. For this reason we had to resort to the more sensitive three-stage IIM technique.

Woode and Bridger (12) in the British Isles have found serological evidence indicating that the Nebraska calf diarrhea virus or similar agent infects piglets both in the laboratory and naturally in the field. In the work undertaken by us no attempt was made to examine local pigs of various ages for the presence of antibody either to IGV or Nebraska calf diarrhea virus. The possibility that pigs acquire IGV infection under field conditions from a human source must be entertained in view of the problem we encountered with nosocomial infection. On two occasions when our conventional piglets were housed in proximity to one another, the control piglet acquired IGV infection. Flewett et al. (6) have witnessed nosocomial IGV infection in a long-stay children's ward. Hospital-acquired IGV infection has also been encountered in our own institution. The control of outbreaks on four separate infant ward areas in our hospital could only be achieved through strict isolation measures. These observations in piglets and infants underscore the ready transmissibility of IGV from infected cases to susceptible recipient.

When IGV-infected animals were injected subcutaneously with [$^3$H]uridine but not with [$^3$H]TdR, labeled virus was obtained. This result not only indicates that IGV possesses a ribonucleic rather that a deoxyribonucleic acid genome, but also provides additional proof that actual virus replication occurred in the piglets. Furthermore, purified virus from infected piglets had a buoyant density in CsCl of 1.36 g/ml. This same buoyant density figure was regularly obtained when we purified virus from stools of IGV-infected infants (M. Petric, M. T. Szymanski, and P. J. Middleton, Intervirology, in press).

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