Enterovirus Replication in Porcine Ileal Explants

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SUMMARY

Organ explants of porcine ileum were cultured in different media for up to 48 h. Tissue preservation was evaluated by light microscopy and by transmission and scanning electron microscopy. Cellular structure was well maintained after incubation for 48 h in CMRL-1066 supplemented with insulin and cortisone. Explants of absorptive or lymphoid tissue from young or adult pigs were incubated with either coxsackievirus B5 (which is infectious for swine) or human poliovirus type 1 (which served as a control) for 24 h at 37 °C. Progeny virus was detected by plaque assay. Replication was most evident in the absorptive tissue explants from young pigs. In tissues from adults, replication occurred equally well in absorptive and lymphoid tissues. Infection in explants was inefficient, and the yield of progeny virus was low.

Intestinal explant cultures are a means of maintaining tissue in vitro under conditions that are relevant to the in vivo state. Viability is best preserved by culturing the tissue at the surface of the medium (Browning & Trier, 1969). Although this technique is suitable for intestinal explants from various host species, different culture media must be evaluated for each species (Trier, 1980). Since the gastrointestinal tract of the pig is similar in structure and susceptibility to that of humans, pigs are a desirable animal model for studying enteric viruses. However, the explant procedure described by Browning & Trier (1969) has not yet been adapted to porcine tissue.

Virus replication in explant culture has been demonstrated for several animal viruses, including porcine enteroviruses (Derbyshire & Collins, 1971; Jensen & Cliver, 1984). In at least some of these reports, however, much of the replication was probably not relevant to virus production in vivo. In these studies, mucosal degeneration was obvious at 24 h and progressed rapidly thereafter (Rubenstein & Tyrrell, 1970). Since the maximum titre was obtained on day 6 post-inoculation, the virus was replicating in the modified epithelium that had replaced the original one. The replication period should therefore be limited by the duration of tissue preservation.

An unresolved controversy concerns whether the enteroviruses replicate in absorptive or lymphoid tissue (Peyer's patches) of the small intestine (Bodian, 1957; Sabin, 1956). We investigated this question using human coxsackievirus B5 (CB5, strain Falk-3) in porcine explant cultures. CB5 causes encephalomyelitic lesions in swine, but does not cause clinical illness (Monlux et al., 1975). Poliovirus type 1 (PO1, strain CHAT) which does not infect pigs, was used as a control. We evaluated the preservation of porcine ileum during 48 h of culture in different media by light microscopy and by scanning and transmission electron microscopy (SEM and TEM, respectively). After selecting the medium best suited to porcine tissue, explant cultures of absorptive or lymphoid tissue were incubated with CB5 or PO1 for 24 h. Viral

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progeny were detected by plaque assay in BGM cells. In addition, virus harvests from explants incubated with PO1 were inoculated onto swine testicle cells to screen for pre-existing infection with an adventitious virus.

Intestinal tissue was acquired from female Yorkshire pigs either 4 to 6 weeks old or 9 to 11 months old. The caudal 30 cm of ileum was split lengthwise and rinsed in five changes of saline containing 270 units/ml penicillin G, 400 μg/ml streptomycin, and 60 μg/ml tetracycline at 4 °C. Explants (4 mm²) were of absorptive or lymphoid tissue (the distinction was visible by eye) and cultured in the presence of 95% O₂/5% CO₂ as described by Browning & Trier (1969). Explants were maintained for up to 48 h, with a renewal of medium after 24 h. In all experiments, uncultured control tissue was immediately placed into fixative for subsequent histological examination.

Explants were cultured in one of the following media (Gibco): CMRL-1066, Trowell T8, Dulbecco's modified Eagle's medium (DMEM) with HEPES buffer, and NCTC-135. Media were supplemented with antibiotics as in the saline, 0-292 mg/ml L-glutamine and 5% foetal calf serum. Hydrocortisone hemisuccinate (0-1 μg/ml) and bovine insulin (1-0 μg/ml) were added to the CMRL-1066 (Sanefuji et al., 1978). Because initial results suggested that the hormones were beneficial, they were added to all the media.

After the culture period, tissues were floated in 0-5% N-acetyl-L-cysteine (Sedlock & Deibel, 1978) for 5 min to facilitate removal of mucus. Those prepared for electron microscopy were fixed in 4% glutaraldehyde at 4 °C for 4 h with gentle rotation, post-fixed with osmium tetroxide, and dehydrated in a series of graded alcohols. Some tissues were embedded in Epon for TEM. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed with a Hitachi HU12 microscope. Other tissues were prepared for SEM by critical point drying, gold-shadowed, and viewed with a Jeolco JSM-U3 microscope. Tissues examined by light microscopy were embedded in paraffin, sectioned at 8 μm, and stained with haematoxylin and eosin. At least eight explants from different culture dishes were examined.

Six explant cultures of each type of tissue from two pigs were inoculated with 10³ to 10⁴ p.f.u./ml of either CB5 or PO1. Cultures were maintained for 1 h at 37 °C with rocking. They were rinsed in five changes of saline and placed in fresh culture dishes with virus-free medium for 24 h at 37 °C. Progeny were harvested from the explants by incubating the tissue and culture medium in 1%, SDS at 37 °C for 60 min, followed by sonication for 30 min. Progeny were assayed in cell culture. The results were examined by analysis of variance. As a control of carry-over inoculum, the virus in some explants was harvested immediately after inoculation and rinsing.

Histological examination of absorptive and lymphoid tissue explants verified that the two types of tissue had been correctly identified. As expected, the germinal centres of the Peyer's patches were much larger and more developed in tissues from young animals than from adults. No other age-dependent differences were visible. In evaluating tissue preservation, we focused attention on the mucosal surfaces of the explants. This was done because early signs of degradation are most evident at the apical regions of absorptive epithelial cells, and because the epithelium is the likely site of virus penetration. Since there is substantial evidence that maintenance of normal metabolism accompanies morphological preservation (Berteloot et al., 1979; Ferland & Hugon, 1979; Kagnoff et al., 1972), structural integrity is a valid criterion of successful culture.

Within a few hours of culture, explants were covered with a thin, translucent coat of mucus. In poorly preserved cultures, mucus was particularly viscous and formed a tight seal over the villi. In all cases, cultured villi appeared shorter and wider than uncultured controls, indicating that the rate of mitosis within the crypts had begun to decrease.

Villous architecture was well maintained in CMRL-1066 supplemented with insulin and cortisol for up to 48 h (Fig 1 a and b). Normal cell desquamation could be seen at the tips of the villi. The microvilli remained uniformly long and closely packed, and strong microfilaments were visible through the terminal web. The integrity of intercellular and basement membranes was unaffected. The cytoplasmic organelles were normal in appearance and distribution. In contrast, substantial cell necrosis was evident in explants incubated for 48 h in either Trowell T8, NCTC-135, or DMEM-HEPES (Fig 1 c).
In virus replication experiments, explants were incubated for 24 h to limit the production of progeny to (at most) three replicative cycles. The percentage of inoculum that remained associated with the tissue following inoculation and rinsing was used as a baseline against which virus replication was detected. Data acquired for carry-over virus and viral progeny were analysed by logarithmic transformation using the SAS statistical package (SAS Institute, Cary, N.C., U.S.A.).

In general, non-specific uptake of PO1 represented about 4 to 7% of the inocula (Fig. 2). There was little non-specific loss of infectivity during the replication period. Carry-over for CB5 was approximately 2 to 4%, lower than that observed for PO1. This apparent loss of virus suggested an eclipse period. This effect was observed with CB5 in both lymphoid and absorptive tissues originating from both young and adult animals.

Virus replication was measured in terms of the percentage of the inoculum recovered after the 24 h incubation period (Fig. 3). As expected, PO1 titres before and after 24 h were equivalent, about 4 to 8% of the inocula. In contrast, CB5 titres increased considerably during the incubation period, particularly in the absorptive explants of young pig ileum, where a mean of 73% of the inoculum was recovered. In contrast, only 11% of the inoculum was harvested from lymphoid tissue explants from young animals. Nevertheless, this result was significantly different (P < 0.05) from the amount attributable to carry-over inoculum. In young animals, therefore, replication of CB5 seemed to occur principally in the absorptive tissue. Replication in the lymphoid tissue took place to a real but limited extent. Results of the experiments using adult
pig ileum were more difficult to interpret. Approximately 34 to 36% of the inoculated CB5 was recovered from both types of tissue after 24 h. This was substantially greater than carry-over inocula, yet less than that recovered from the absorptive tissue of young pigs. This result suggested that replication had occurred in both types of explants. There was no statistical difference between the two tissue types (P > 0.05).

In this study, replication of CB5 was clearly evident in absorptive tissue explants from young animals. The ratio of progeny to carry-over virus was about 31. A lesser amount of replication occurred in the lymphoid tissue. However, this limited progeny virus might have been produced in the absorptive villi which exist between germinal centres of the Peyer’s patches. In explants of adult ileum, CB5 replicated equally well in absorptive and lymphoid tissues. Each type produced approximately half as much progeny as had been detected in absorptive tissue explants from young pigs. Differences based on animal age were surprising, since the 4-week-old pig’s intestine is believed to be functionally equivalent to the adult’s. Our results suggest that there may be subtle differences in number or function of the virus-susceptible cells. In humans, children are more likely to be infected with enteroviruses than are adults for a variety of reasons, including lack of previous exposure and generally poor sanitary habits.

Finally, these results suggest that infected porcine ileal cells produced a small number of progeny virions, approximately $10^{5}$ p.f.u. per absorptive tissue explant from young pigs. If the virus yield per epithelial cell was similar to that from cultured cells (about $10^{2}$ p.f.u. of PO1 per HeLa cell; Howes, 1959), there may have been only $10^{3}$ infected cells per explant. Since there were roughly $3 \times 10^{5}$ absorptive cells per explant, only one in 300 absorptive cells became infected. This inefficiency may help explain the lack of gastrointestinal symptoms that is characteristic of enteroviral infections. Studies that identify the susceptible cells present on ileal explants will further elucidate this issue.

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