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ENTEROVIRUS REPLICATION IN ILEAL EXPLANTS

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SUMMARY

Porcine enterovirus 3 replicated in explants of fœtal pig intestine, as was shown using photosensitization with neutral red to distinguish between inoculum and progeny virus. Progeny virus titres reached $10^5$-$10^6$/ml with 2-4 days' incubation.

Key-words: Enterovirus, Replication, Ileum; Explant, Fœtal pig.

INTRODUCTION

Most studies to date of the interactions of human and other animal viruses with their host cells have been conducted in cells cultured in vitro. Little has been learned of how viruses initiate infections in cells of the host organism because the target cells are not readily accessible to study in vivo.
and tend to lose their differential properties when separated, for culture, from the tissue milieu. Receptor specificities, for example, are sometimes modified in this way, such that cells cultured in vitro in monolayers will support replication of viruses to which they are not susceptible in vivo. We wanted a system with which to study the early interactions of enteroviruses with cells of the ileum, which appears to be the site at which most enterovirus infections are initiated in vivo [1]. Explant cultures, in which the component cells are retained in their normal relationships with each other in the tissue, were examined for this purpose.

The use of explant cultures for the detection and study of viruses has been reported in recent years [2-5, 7-9, 11]. Since a close analogy exists between the porcine and human digestive tracts and their respective enteroviruses, use of porcine intestinal explants with porcine enteroviruses provides an animal model system relevant to enterovirus infection of human intestines. This report describes replication of porcine enterovirus 3 (PE3) in an explant system from fetal pig intestine, using photosensitization with neutral red (NR) to distinguish between inoculum and progeny virus [10].

**MATERIALS AND METHODS**

**Cell cultures.**

Mini-pig kidney (MPK) cells, a continuous line obtained from the American Type Culture Collection, were maintained in Eagle’s minimal essential medium with non-essential amino acids, plus 20% fetal calf serum (FCS) and 100 U/ml of penicillin G sodium and 100 µg/ml of dihydrostreptomycin sulphate, buffered to pH 6.8 with NaHCO₃. Monolayers were grown in 25-cm² polystyrene flasks (Falcon) at 37°C.

**Virus.**

PE3, strain ECPO-6, was obtained from Dr E. H. Bohl, Ohio Agricultural Research and Development Station, Wooster, OH. Virus titre was determined by cytopathic effects (CPE) produced in MPK. Virus was diluted serially in Dulbecco’s phosphate-buffered saline containing 2% FCS and antibiotics as in the cell culture medium; and duplicate cultures were inoculated with 0.5 ml of each dilution, incubated at 37°C for 7 days, and scored for CPE. The titres were calculated as most probable numbers of cytopathic units (MPNCU) by the formula of Thomas [6], and expressed as the log₁₀ of this number, to two significant digits.

Virus was sensitized with NR by a modification of procedures described for poliovirus [10]. Manipulations were done under a red safety lamp (Safelight Model OC, Eastman Kodak). All monolayers in medium containing NR were incubated

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CPE = cytopathic effect.
FCS = fetal calf serum.
HBSS = Hanks' balanced salt solution.
MPNCU = most probable number of cytopathic units.
MPK = mini-pig kidney.
NR = neutral red.
PE = porcine enterovirus.
PFU = plaque-forming unit.
in the dark. PE3 stock (5 × 10^5 plaque-forming units in 0.5 ml) was inoculated into MPK cultures that had been incubated for 55 h at 37 °C with medium containing 5 μg/ml NR. The monolayers were rocked for 60 min in the dark, the inoculum was decanted, the monolayers were rinsed twice with medium containing NR, fresh medium containing NR was added, and the monolayers were incubated for 72 h at 37 °C in the dark. At 72 h, one of the cultures was examined and showed an advanced stage of CPE. The remaining cultures were frozen at −20 °C and thawed at room temperature three times, and stored in the dark at 4 °C. The pooled suspension (titre = 1 × 10^8/ml) was diluted 100-fold in NR medium and passed a second time in the presence of NR. Virus from the second passage was stored in the dark at 4 °C.

Two samples of this PE3 stock were diluted 100-fold into 0.03 M phosphate buffer, pH 7.1. One sample was placed in a test tube 15 cm from two 15-watt fluorescent bulbs and light-shocked for 60 min at room temperature. The titres of the samples were 1 × 10^8/ml without and 1 × 10^6/ml with light-shocking, a 1 × 10^5-fold difference.

Explants.

Explants were prepared from portions of lower ileum excised aseptically from fœtal pigs 22-30 cm in length within 50 min after death. Pieces 2.5-5 cm in length were immediately placed into Petri dishes containing chilled Hanks' balanced salt solution (HBSS) and held on ice. The intestinal segments were opened longitudinally and rinsed gently with HBSS to remove meconium debris. Segments approximately 2-3 mm square were cut with scalpels, then gently placed with forceps, mucosal side up, onto prescored areas in 60 × 15-mm Petri dishes that had been incubated overnight in a moist chamber at 37 °C. Five explants per dish were placed at positions roughly equidistant from each other. In each experiment, an equal number of control dishes, which had been incubated overnight in the moist chamber but into which no explants were placed, was also used. Immediately after placement of the explants, all dishes were removed to the dark and further manipulations done under a red safety lamp.

The photosensitized virus stock was diluted 100- or 1,000-fold, and 0.1 ml was dripped onto each explant; 0.5 ml of the same suspension was inoculated into each control dish. All dishes were incubated in the moist chamber in the dark for 3 h at 37 °C. Then 1.3 ml of fresh medium was added to each dish (total volume, 1.8 ml per dish), and the dishes were placed into the moist chamber and incubated in the dark at 37 °C in 5% CO₂ in air. Both explant cultures and control dishes received Eagle's medium with 0.2% bovine serum albumin and antibiotics as in the cell culture medium, pH 6.8-7.0. At 2, 4 and 6 days, all of the medium (1.8 ml) was harvested from each dish, and 1.8 ml of fresh medium was added. In a typical experiment, three dishes of explants and three control dishes were prepared. Harvested medium from the three explant dishes was pooled, as was that from the three control dishes, on each collection day. All harvested medium was stored at 4 °C in the dark. The titres of light-resistant virus inoculated on day 0 were determined by adding 0.5 ml of inoculum to 1.3 ml of medium and holding this mixture at 4 °C in the dark until light-shocking (experiments A and E) or by harvesting the inoculum, with medium, from an extra set of plates with and without explants at the end of the 3-h initial adsorption period (experiments B, C and D). Day 0 samples and medium harvested from experimental and control dishes were diluted 100-fold in phosphate buffer (pH 7.1), light-shocked, and assayed.

Explants were also prepared for microscopic observation. In a typical experiment, six dishes of explants were prepared, only three of which were inoculated with virus. All dishes were incubated as described previously. On days 2, 4 and 6, one inoculated and one uninoculated dish were removed and observed under a dissecting microscope for explant attachment and villous structure.
RESULTS

Replication of PE3 in explants was demonstrated in five experiments (fig. 1). The titres of the light-resistant virus peaked on day 2 or 4 with maximal yields of $1 \times 10^4$ to $1 \times 10^6$ MPNCU/ml and average yields of $5 \times 10^4$ to $1 \times 10^5$ MPNCU/ml. Attachment of explants to scored areas of the petri dishes remained good (60-100\%) for 4 days and then declined; similarly, villous structure appeared typical for 4 days, but by 6 days there was shortening and thinning of villi and some buildup of mucus on the surface of the explants. Differences between virus-inoculated and uninoculated explants were not perceptible.

![Graph showing virus titres over time](image)

**Fig. 1.** -- *Titres of light-resistant virus as a function of time at 37°C in five experiments.*

The open portion of each bar shows the titre of the virus incubated with ileal explants, and the filled portion of the bar shows the titre of the same quantity of virus incubated in dishes without tissue (controls). Where only a filled bar appears, only a suspension without tissue was tested (experiments A and E) or the titres of the two suspensions were the same (experiment C). Arrowheads pointing downward indicate that the control suspension gave a negative test result at the lower limit of virus detection. The photosensitized virus suspension had been diluted 100-fold initially in experiments A, B, and C, and 1,000-fold initially in experiments D and E.

DISCUSSION

It is clear that substantial replication of the virus took place in these explant cultures, for the yields of light-resistant virus were considerable and were slightly higher than expected on the basis of *in vivo* data. Perorally infected 4-week-old pigs shed PE3 virus at levels of $1-4 \times 10^4$ PFU/g of stool, with an average daily faecal output of 25 g [unpublished data]. The ileal tissue present in one dish of 5 explants is estimated to be 0.13-0.15\%
of the total quantity of ileal tissue in a 4-week old pig, so we calculated that one dish should yield $1-2 \times 10^4$ MPNCU/ml in 2 days.

Replication of PE3 virus proceeded to substantial titres in the ileal explants without perceptible destruction of the epithelium, which is probably to be expected of an enterovirus which, in vivo, replicates in the intestine without causing overt signs of gastroenteritis. The replication of the virus to peak titres within 2-4 days is also expected in view of the short replicative cycle of this virus (12 h at $37^\circ C$).

Intestinal organ cultures have been used by other investigators to study virus replication. Coronavirus have been shown to replicate in organ cultures of pig and bovine intestine [2, 9]. Echovirus 11, a human enterovirus that causes gastroenteritis, has been studied using human foetal intestinal explants [5, 8, 11]. Derbyshire and Collins [3] studied Talfan virus, a porcine enterovirus that does not cause enteritis, and found no substantial virus replication in explants of small intestine until 6-8 days after inoculation; more importantly, virus replication could not be demonstrated consistently and appeared to take place after structural degeneration of the epithelium (which occurred within 48 h), presumably in the lamina propria. Rubenstein and Tyrrell [8] reported replication of poliovirus 1 to $1 \times 10^4$-1 × $10^5$ PFU/ml in explants of human foetal ileum after 6 days' incubation; however, the virus continued to grow to maximal titres in the explants after the original intestinal epithelium disappeared. Wyatt [11] reported replication of poliovirus type 1 in human foetal small intestinal explants, with peak titres of more than $1 \times 10^6$ PFU/ml after 6 days; no distinction was made in this study between explants of the ileum and other segments of small intestine, and, surprisingly, virus replication was associated with destruction of the epithelium.

In this study, we have used photosensitized virus to distinguish input virus from progeny virus. Other methods for verifying virus replication, such as net virus output, immunofluorescence [5] and electron microscopy [11], do not allow confirmation of enterovirus replication until 6-8 days after infection, i. e. until substantial virus replication has occurred. The use of photosensitized virus allows detection of even low levels of early virus replication (within 48 h) in explant cultures.

Porcine intestinal explants offer a method of studying cell-enterovirus interactions using an animal system (pig) which is closely analogous to the human gastrointestinal system. They offer an advantage over other animal systems and a source of data relevant to human systems without the problems of obtaining human tissue. The use of photosensitized virus allows detection of low levels of early virus replication (within 48 h) in explants and will be valuable in quantitative studies of the initial events in viral infection and replication. Explant culture systems will also allow identification of enterovirus receptor sites in the ileum and study of the early interactions of enteroviruses with receptors on susceptible target cells.

The present explant culture system would not permit maintenance of cultures for long enough periods of time to model the entire course of an
enteric viral infection, but it does seem to preserve the structure and function of the explants sufficiently during the first 4 days to allow studies of how intestinal infections are established and for substantial viral replication to proceed. In particular, this technique might permit comparison between various segments of the intestine as to their ability to serve as the initial site of infection; this is a question which has been much discussed but not totally resolved. Such cultures may also serve eventually to explain the apparent inefficiency with which enteroviruses initiate infection in vivo.

RÉSUMÉ

Réplication des entérovirus sur explants iléaux

Une technique de photosensibilisation à l'aide de rouge neutre pour distinguer les virus de l’inoculum des virus néoformés, a permis de montrer qu’un entérovirus porcin de type 3 se réplique dans des explants intestinaux de fœtus porcin. Les titres des virus néoformés atteignent $10^5$ à $10^6$/ml après 2 à 4 jours d'incubation.

MOTS-CLÉS : Entérovirus, Iléon, Réplication ; Explant, Fœtus de porc.

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