Coxsackievirus-cell interactions that initiate infection in porcine ileal explants

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Summary. Coxsackievirus B 5 (CB 5) labeled with tritiated uridine was used to trace the interaction of the virus with explant cultures of porcine ileum. Similarly labeled human poliovirus 1 (PO 1), which is not specifically retained by porcine tissue, was used as a control. The explant procedure employed could maintain ileal tissue in a differentiated state for up to 48 hours. Porcine ileum was acquired from both young (4–6 week-old) and adult (9–11 month-old) animals. Inoculated explants of either absorptive or lymphoid tissue were incubated at temperatures selected to permit either viral adsorption or penetration and elution to occur. Retention of radioactive virus was quantitated by liquid scintillation counting and localized by autoradiography. Only in absorptive tissue explants from young animals did adsorption of CB 5 at 6 °C exceed penetration at 37 °C. This suggested that incubation at 6 °C may not be an appropriate condition for studying enterovirus adsorption in explants. CB 5 penetrated most efficiently into lymphoid tissue explants from young animals, indicating that these tissues could discriminate between CB 5 and PO 1. In explants from adults, CB 5 penetrated equally well into lymphoid and absorptive tissues. Virus penetrated into the absorptive epithelial cells and, possibly, the lamina propria near the villous tips. Low efficiency of penetration, and the non-critical function of these target cells, may help account for the characteristic lack of gastrointestinal symptoms in enterovirus infections.

Introduction

Although the early interactions between enteroviruses and cultured cells have been studied in detail, almost nothing is known about how these viruses initiate infection in vivo. The primary site of replication is believed to be the ileum [2, 16, 27], but the identity of the susceptible cells remains uncertain. In part, this

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is because the intestinal infection is completely inapparent. There are no histological changes and there are no gastrointestinal symptoms, even though a great deal of virus is shed for as long as 6 weeks.

Classic attempts to locate receptors in the small intestine produced conflicting results. Bodian [3] concluded that the enteroviruses replicate in aggregated lymph nodules (Peyer's patches), and are shed into the lumen within lymphocytes. In contrast, others [27, 28] maintained that the absorptive mucosa was the site of virus-cell interactions. More recently, the intestinal tracts of monkeys fed poliovirus 1 were examined using immunofluorescence [15]. This study suggested that virus had been replicating within endothelial cells of the lamina propria. However, the specificity of some of the fluorescence observed was seriously questioned. The matter remains unresolved.

In our study, porcine ileal explant cultures were incubated with ^{3}H-labeled coxsackievirus B 5 (CB 5) or poliovirus 1 (PO 1) under conditions that permitted viral adsorption, or penetration and elution, to occur. CB 5 causes encephalomyelitic lesions in swine, but does not cause clinical illness [18]. PO 1, which does not infect pigs, was used as a control. Viral retention was quantitated by measuring radioactivity, and localized by an adaptation of a very sensitive method of autoradiography [23].

Methods

Cells and media

BGM cells were obtained from Flow Laboratories (McLean, VA). Monolayer cultures were grown in Eagle's minimum essential medium containing 10 per cent fetal calf serum as previously described [11].

Virus assays

PO 1 (vaccine strain CHAT) was obtained from the American Type Culture Collection. CB 5 (strain Falk-3) was acquired from the Wisconsin State Laboratory of Hygiene. Both viruses were passaged several times in BGM cells. Virus stocks were harvested and plaque assays were conducted as previously described [11].

Preparation of radioactive viruses

Radioactive CB 5 (^{3}H-CB 5) and PO 1 (^{3}H-PO 1) were replicated in the presence of ^{3}H-uridine and purified by column chromatography as described [11]. Five or six peak fractions were pooled and further purified using a microconcentrator unit (Millipore Corp., Bedford, MA) with a molecular weight cut-off of 30,000. The preparation was rinsed three times. The specificity of radioactive labelling was determined by measuring loss of radioactivity after filtration through a cellulose nitrate membrane [11].

Preparation of explant cultures

Female Yorkshire pigs were deprived of food overnight and killed by exsanguination. Young animals were 4–6 weeks old, and adults were 9–11 months old. The caudal 30 cm of ileum was slit lengthwise and rinsed in five successive changes of saline containing 270 U/ml penicillin-G, 400 µg/ml streptomycin, and 60 µg/ml tetracycline. All buffer solutions were kept in an ice bath. Explants (4 mm^2) of absorptive or lymphoid tissue (the distinction is visible grossly) were cultured in 95 per cent O_2/5 per cent CO_2 using the explant technique

described by Browning and Trier [4]. All layers from mucosa to serosa were included in the explants. The culture medium comprised CMRL 1066 (Grand Island Biological Co., Grand Island, NY) supplemented with antibiotics as in the saline, fetal calf serum, L-glutamine, insulin and cortisone as described [10]. In each experiment, three to five explants maintained in uninoculated medium were placed into Carnoy's fixative (3 parts ethanol and 1 part glacial acetic acid) immediately after the incubation period, and rocked at 4 °C for 4 hours. Paraffin-embedded sections were stained with hematoxylin and eosin. Tissue preservation was evaluated by light microscopy.

Virus adsorption and penetration into explants

Tissue explants were incubated with rocking in medium containing 10^4–10^5 PFU/ml of ^{3}H-CB 5 or ^{3}H-PO 1. In each experiment, the level of radioactivity was approximately equal, generally 1,000–1,300 disintegrations per min (DPM)/ml. The preparations of CB 5 had approximately 145 PFU/DPM, and those of PO 1 had 40 PFU/DPM. The particle/PFU ratios, as determined by electrophoresis and scanning electron microscopy, equaled 38 for CB 5 and 447 for PO 1 [11]. Therefore, explants were inoculated with either 5.5×10^6 particles of CB 5 or 1.8×10^7 particles of PO 1.

Viral adsorption was measured by incubating the explants for 6 hours at 6 °C. Viral penetration and elution were investigated by incubating the explants for 1 hour at 37 °C. Following the incubation period, explants were rinsed vigorously in five changes of saline (containing antibiotics) to remove unattached virus. Radioactivity retained by each explant was quantitated by digestion in 0.75 ml of Protosol (New England Nuclear, NEN, Boston, MA) at 55 °C for 22–24 hours. The sample was decolorized by adding 50 µl of hydrogen peroxide, heated for 30 minutes at 55 °C, and cooled. Radioactivity was counted in 10 ml of Econofluor (NEN) using an LS 5,800 counter (Beckman Instruments, Inc., Fullerton, CA).

The phenomena of viral adsorption and penetration were each analyzed using ileal tissue from two young pigs and two adults. A total of eight animals were involved. Thirty absorptive tissue explants and 30 lymphoid tissue explants were prepared from each animal. These were incubated with either ^{3}H-CB 5 or ^{3}H-PO 1. Results from the replicate explants were examined by analysis of variance using the SAS statistical package (SAS Institute, Inc., Cary, NC).

Autoradiography

Retained virus was localized by liquid emulsion autoradiography. Explants inoculated with either ^{3}H-CB 5 or ^{3}H-PO 1 were preserved in Carnoy's fixative and embedded in paraffin. Sections 8–10 µm thick were mounted onto glass slides precleaned with absolute ethanol. The slides were heated to 60 °C for 1 hour, cooled overnight, and degreased by soaking in two changes of Histo-clear (National Diagnostics, Somerville, NJ) for 30 minutes each. The slides were rehydrated by soaking for 3 minutes each in decreasing concentrations of ethanol, rinsed in distilled water, and allowed to dry overnight. All subsequent steps were performed in complete darkness. Slides were dipped into undiluted NTB-3 emulsion (Eastman Kodak Co., Rochester, NY) liquified in a 40 °C water bath, and dried in an upright position. They were placed into a solution of toluene (scintillation grade) containing 5 g/l of 2,5-diphenyloxazole (PPO) and 0.1 g/l of p-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP). The slides were sealed in a black rubber tank and exposed at –20 °C for 1, 2, 3, or 4 days. Slides were developed in Dektol (Eastman Kodak), and stained with cresyl violet [29].

Results

The percentage retention of radioactive virus by each explant was defined as (net DPM retained/net DPM inoculated) × 100, using uninfected explant tissue as a blank for ^{3}H counting. The values reported in Figs. 1 and 2 are geometric

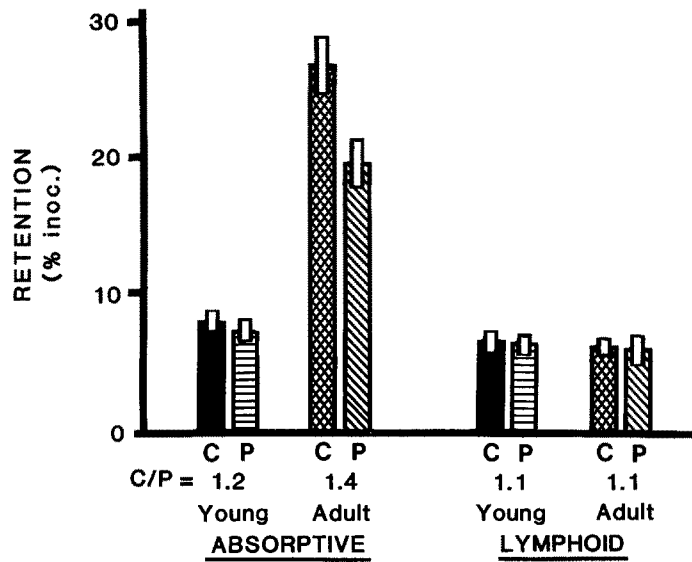**Fig. 1.** Adsorption of CB 5 (*C*) and PO 1 (*P*) to porcine ileal explant cultures during 6 hours at 6 °C; bars: +/–2 S.E.M.
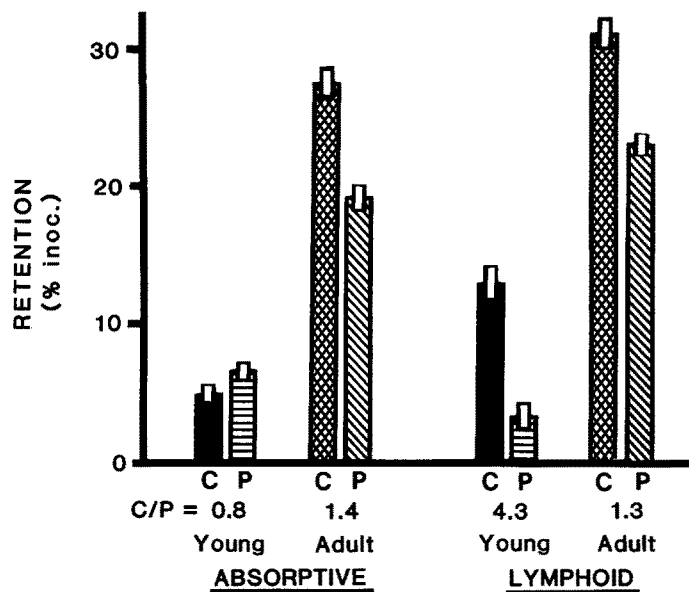

Fig. 2. Penetration of CB 5 (*C*) and PO 1 (*P*) into porcine ileal explant cultures during 1 hour at 37 °C; bars: +/–2 S.E.M.

means of the 30 replicate explants from 2 pigs. The ratios determined by dividing the retention of CB 5 by that of PO 1 reflect the relative amounts of specific versus nonspecific interactions.

Virus adsorption into ileal explants

Specific adsorption of CB 5 was most evident on absorptive tissue explants from adult animals (the CB 5/PO 1 ratio was somewhat higher for this tissue than

for any other). However, both specific and nonspecific adsorption were high in this tissue. The reason absorptive tissue from the ileum of adult animals might retain large numbers of virions was not readily apparent. The ratios of specific to nonspecific retention for the other three types of explants were approximately equal.

Statistical analysis of virus adsorption at 6 °C indicated that the type of virus in the inoculum was significant (P < 0.0001). The effects of animal age (P = 0.0270) and type of tissue (P = 0.0233) were also significant factors. Animal-to-animal differences were more than 10 times greater than differences among replicates originating from the same tissue. Differences between the two tissue types were 20 times greater than those among replicates.

Virus penetration into ileal explants

The results for virus penetration into ileal explants indicate a different pattern of retention than was seen for adsorption. CB 5 was clearly preferentially retained by the lymphoid tissue of the young animal (CB 5/PO 1 > 4). Moreover, nonspecific uptake was extremely low, only 3 percent of the inocula. This result suggests that lymphoid tissue from young pigs can distinguish between homologous and heterologous viruses.

In contrast, slightly more PO 1 than CB 5 was retained by absorptive tissues of young animals. In both types of tissue from adult animals, penetration of CB 5 exceeded that for PO 1. However, the difference between the two viruses in these animals was much less dramatic. Unexpectedly, CB 5 was extensively retained in both lymphoid and absorptive tissues from the adult animals.

Statistical analysis of virus penetration at 37 °C indicated that the factors of type of virus and age were significant (P < 0.0001 and P = 0.0010, respectively). The type of tissue was not significant (P = 0.0783). The variance among animals (0.113) was considerably higher than that among replicative explants (0.015).

Virus elution from receptors in ileal explants

We had hoped that the extent to which viruses elute off of cell membrane receptors after adsorbing to them could be estimated indirectly by comparing virus retention at 6 °C to that at 37 °C. However, experimental conditions appropriate to quantifying enterovirus adsorption in cell culture did not seem to be appropriate in explants. Lymphoid tissue retained considerably more virus at 37 °C than at 6 °C (with the unexplained exception of PO 1 retention in young pigs). Similarly, virus adsorption and penetration into absorptive tissue explants were equivalent in all cases except for the interaction between CB 5 and tissues from young pigs. Because an earlier study showed that the absorptive tissue explants of young pig ileum support replication of CB 5 [10], this observation may be noteworthy.

Autoradiography

Most of the tissue incubated with virus was free of radioactivity. This result was not surprising, since both the amount of radioactivity in the inocula and the efficiency of virus retention by explants were low. Retention of radioactive virus was detected as small groups of silver grains (generally 3–15 grains) associated with the tissue. Background was recognized as individual grains randomly distributed through the tissue and in the emulsion not overlying the tissue. Under optimal conditions, tissue sections exposed for 2–4 days were almost totally free of background grains. Silver grains associated with dust, mucus, or knife marks in the section were considered artifactual and disregarded. In some instances, silver grains were clearly deposited within the cytoplasm of mucosal cells. Significantly, this was only observed in tissues incubated at 37 °C. However, since tritium emissions will travel about 2 μm through the emulsion [24], groups of grains outside of the cellular boundaries were also significant. Tissues from both young and adult animals showed the same trends.

Sites of virus adsorption

Absorptive tissue from both young and adult pigs that had been incubated with CB 5 at 6 °C for 6 hours appeared to have adsorbed virus at mucosal cells in two regions of the epithelial surface: very near the crypt openings (Fig. 3) and
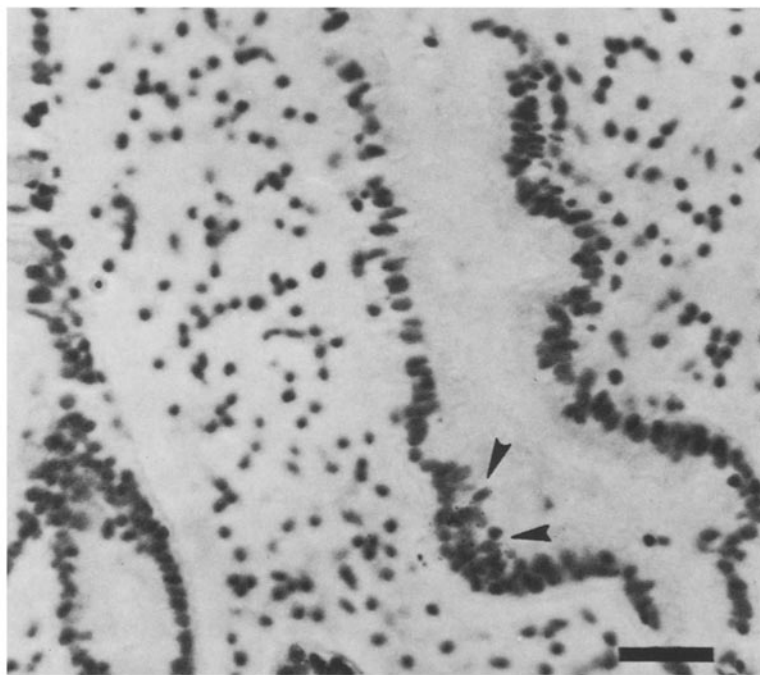

Fig. 3. Autoradiograph showing silver grains (arrows) associated with epithelial cells at the base of a villus on an absorptive tissue explant from adult animal. The explant was incubated with radioactive CB 5 for 6 hours at 6 °C. Exposure: 3 days; bar represents 25 μm

at the upper third of the villi (Fig. 4). It was not possible to determine whether one region contained substantially more grains than the other. Additionally, there was no evidence of silver grains within the lamina propria, even in those instances where the integrity of the villus had been compromised, such as where the mucosal layer had been torn. Absorptive tissue incubated with PO 1 exhibited fewer grains than if incubated with CB 5. As expected, those present were dispersed as individual grains rather than in groups.

Lymphoid tissue explants incubated with CB 5 showed accumulations of silver grains at the epithelial layer that were comparable in size and number to those seen in sections of absorptive tissue (Fig. 5). Since it is not possible to distinguish the different mucosal cell types of the Peyer's patches with a light microscope, the identity of these receptor cells could not be determined. Radioactivity was only associated with epithelial cells and had not penetrated into the deeper tissue. This was evidence that exposure to cold temperature had substantially decreased the tissue's normal function of sampling antigens present in the lumen. Explants incubated with PO 1 developed insignificant numbers of silver grains.

Sites of virus penetration

Explants of absorptive tissue incubated with CB 5 showed silver grains at the upper portions of the villi. These appeared either in the mucosal cells near the
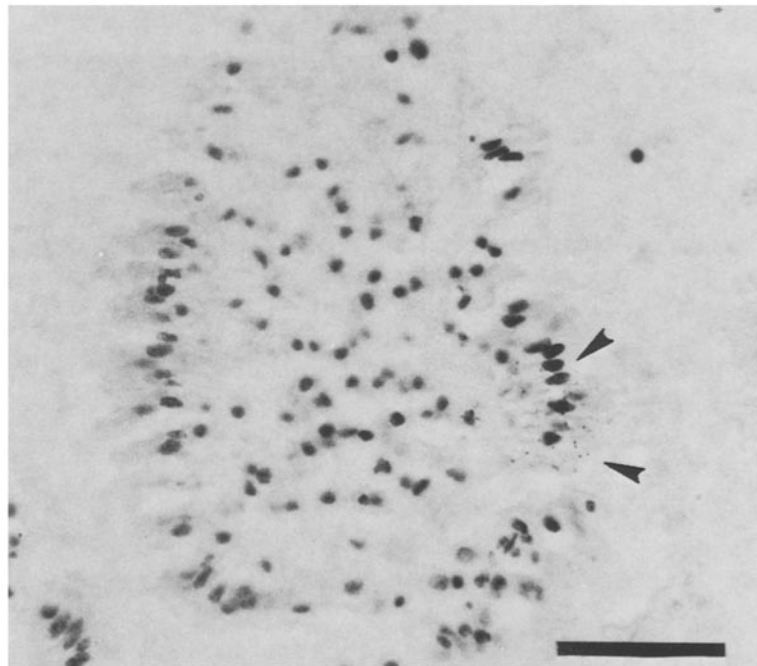

Fig. 4. Autoradiograph showing silver grains (arrows) associated with epithelial cells at the upper third of a villus on an absorptive tissue explant from an adult animal. The explant was incubated with radioactive CB 5 for 6 hours at 6 °C. Exposure: 4 days; bar represents 25 µm
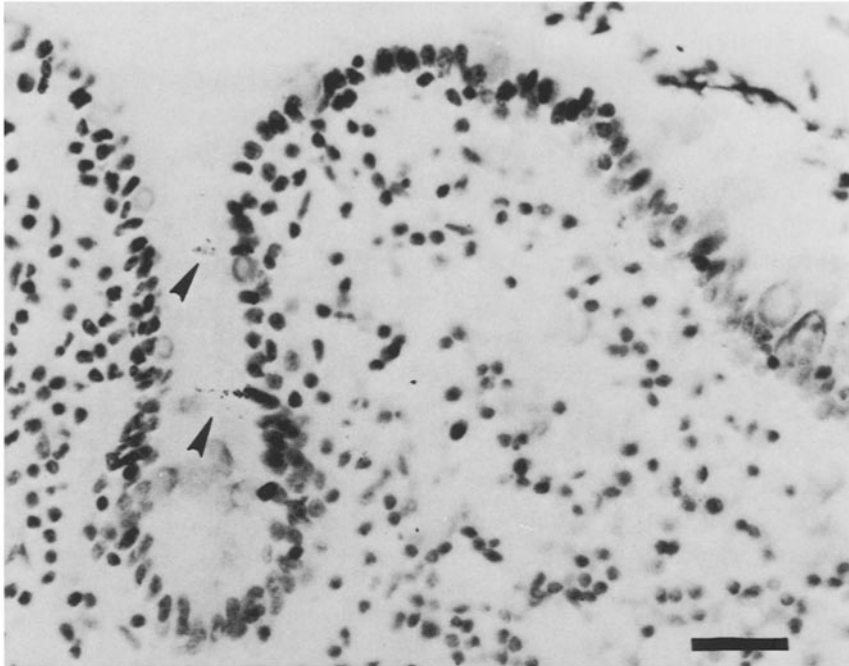
Fig. 5. Autoradiograph showing silver grains (arrows) associated with the epithelial cells overlying a Peyer's patch on an explant from an adult animal. The explant was incubated with radioactive CB 5 for 6 hours at 6 °C. Exposure: 2 days; bar represents 25 μm
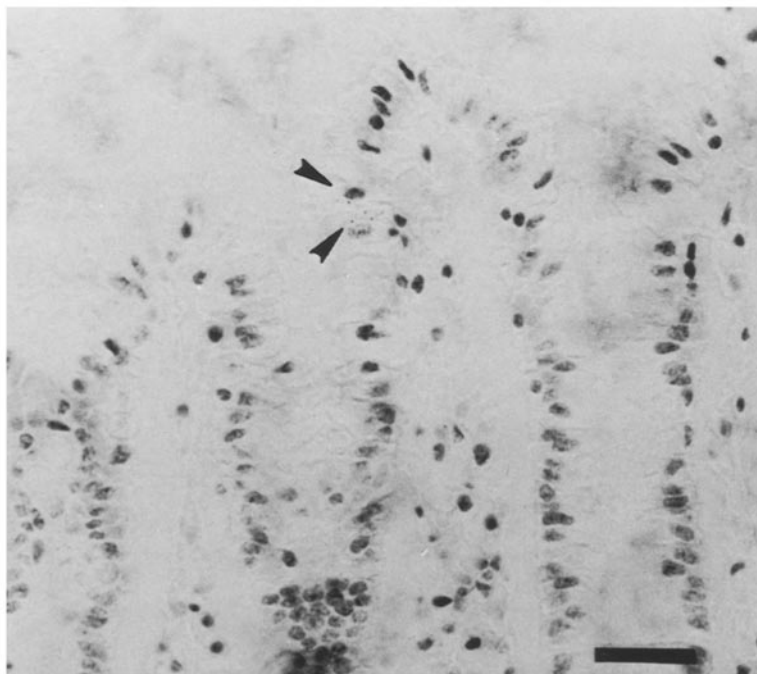

Fig. 6. Autoradiograph showing silver grains (arrows) associated with epithelial cells near villous tips on an explant from an adult animal. The explant was incubated with radioactive CB 5 for 1 hour at 37 °C. Exposure: 4 days; bars represent 25 μm
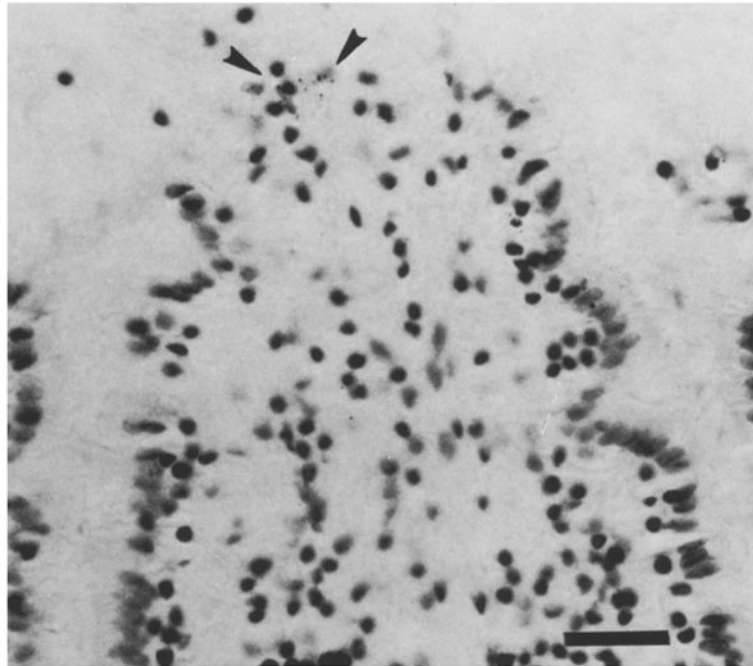
Fig. 7. Autoradiograph showing silver grains (arrows) associated with the lamina propria of a denuded villous tip on an explant from an adult animal. The explant was incubated with radioactive CB 5 for 1 hour at 37 °C. Exposure: 3 days; bar represents 25 µm

villous tips (Fig. 6) or, in some instances where the mucosal cells at the tip had been lost, at the denuded tip or deeper within the lamina propria (Fig. 7). It is impossible to know whether loss of the tip cells was necessary for virus to enter. The cells may have been sloughed during the inoculum period or during embedding. In at least one tissue section, substantial numbers of grains appeared within the lamina propria of a villus whose tip cells appeared intact. However, mucosal cells of the villous tip outside of the plane of the section may have been lost. Once again, PO 1 retention did not follow any noticeable pattern.

Explants of lymphoid tissue incubated with CB 5 produced extensive numbers of silver grains widely distributed through the tissue. This was particularly true of cultures from adult animals. Tissues inoculated with PO 1 also developed large numbers of grains. These results were in accord with the quantitative data acquired. It appeared that the mucosal cells overlying Peyer's patches were actively sampling antigens from the lumen at 37 °C, whereas at 6 °C their activity was considerably less. It was not possible to determine from the autoradiographs whether CB 5 was retained or processed differently from PO 1.

Discussion

This study was designed to investigate enterovirus-cell interactions under conditions that are relevant to the in vivo state. Because isolated intestinal cells dedifferentiate rapidly, we used explant cultures as a compromise between the

in vivo and cell culture systems. We were able to examine the phenomena of virus adsorption and penetration by analyzing the uptake of radioactive virus.

Although the structure and physiology of the porcine small intestine change drastically during the first 2–3 weeks of life [19, 21] the small intestine of 4–6 week-old pigs is generally thought to be equivalent to that of adult pigs. We were therefore surprised that the ages of our animals had a significant influence on virus-cell interactions. The Peyer's patches were much larger and had better defined boundaries in the young animals than in the adults. This is presumably because the response to an antigenic stimulus is more profound in an immunologically inexperienced animal.

In general, CB 5 retention by explants was higher at 37 °C than at 6 °C. Only in absorptive tissue explants of young pig ileum did adsorption of CB 5 at 6 °C exceed penetration at 37 °C. These results were surprising, since they are contrary to classic observations made using cultured cells [5, 6]. Our results suggested that the experimental conditions appropriate to quantifying virus adsorption in cell culture were not appropriate in explants. If so, the sites of CB 5 adsorption identified by autoradiography would not be relevant to virus adsorption in vivo.

CB 5 selectively penetrated the lymphoid tissue explants of young pig ileum (CB/PO 1 = 4.35). This result indicated a high degree of specificity, since these tissues could discriminate between two morphologically similar viruses. Specialized epithelial cells of the Peyer's patches (M cells) are believed to sample the antigens present in the lumen of the intestine and present them to the lymphocytes. Selection of antigens is generally thought to be nonspecific. Substances retained by M cells include ferritin [1], horseradish peroxidase [22], and carbon particles [13]. Reoviruses, known to penetrate into the intestinal epithelium via the M cells [31] did not interact with M cells specifically [30]. However, penetration of reoviruses into other epithelial cells was virus-specific: reovirus 1 interacted with absorptive cells overlying lymphoid tissue but not those covering ileal villi. In our study, CB 5 may have been retained both nonspecifically by M cells and specifically by the absorptive cells of the lymphoid tissue. In constrast, PO 1 uptake may only have been the result of nonspecific interaction with the M cells. This hypothesis could be tested by autoradiography using electron microscopy.

Although we have shown that CB 5 does not replicate extensively within lymphoid tissue explants of ileum from young pigs [10], virus entry into the Peyer's patches is not irrelevant. It is likely that virus enters the systemic circulation by this route. Early studies demonstrated that small numbers of ingested particles such as bacteria [8] and bacteriophage [17] or large molecules such as botulinum toxin [9] routinely pass from the intestinal lumen into the blood. The route of transport seemed to be via the lymphatic system [12].

In adults, virus penetration into the two tissue types was approximately equal. This could suggest that subtle functional or structural differences exist between young and adult tissues. On the other hand, unexpectedly high retention

of PO 1 suggested that the differences observed resulted from nonspecific factors, such as differing amounts of mucus. For example, lymphoid tissue contains fewer goblet cells than absorptive tissue. Because the Peyer's patches were so large in the young animal, explants cut from lymphoid tissue may have been somewhat isolated from the mucus released by the absorptive tissue.

Autoradiographs showed similar trends for animals of both age groups. CB 5 penetrated into the epithelial cells along the upper third of the villi (those at or approaching senescence) and/or into the lamina propria. It was not possible to determine whether the virus gained access to the lamina propria through an intact or compromised epithelial layer. This question might be answered by autoradiography of serial sections. The apparent adsorption of virus to mucosal cells at the bases of the villi did not lead to penetration. It would be interesting to determine whether the virions eluting from these cell receptors had altered protein capsids, as has been observed in studies using cell cultures [6, 7, 14, 25].

In light of our results, the paradox of extensive virus replication without gastrointestinal disease may be related to three factors. First, only a small proportion of ileal tissue interacted with virus. This was reflected in the low efficiency of CB 5 penetration and the infrequency of silver deposits indicating viral presence in autoradiographs. Second, the autoradiographs implicated the aging mucosal cells and/or cells of the lamina propria as the most probable sites of virus penetration and, presumably, replication. These cells play a minor role in the digestion of food and the absorption of nutrients. Any loss of intestinal function due to virus replication is therefore unlikely to cause intestinal disease. Third, the infected cells apparently produced a low number of progeny viruses [10]. Furthermore, the prolonged period of virus shedding could be explained by the large number of potentially susceptible cells and the rapid renewal rate of the mucosal cells (about 5 days in humans). Varying rates of cell replacement may also help account for the distinction seen between young and adult animals. Because the mitotic rate is slower in younger animals, aging epithelial cells stay attached to the villi longer. It is also possible that porcine mucosal cells of different ages produce varying amounts enterovirus, as has been suggested for transmissible gastroenteritis virus [20].

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