Intracellular Bacteria of Porcine Proliferative Enteropathy: Cultivation and Maintenance In Vitro

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An obligate intracellular bacterium was isolated from the intestines of all 10 cases of porcine proliferative enteropathy from four different pig farms. The organism grew in a rat enterocyte cell line (IEC-18) and was maintained over 20 passages. The growth of the bacteria was assessed by immunostaining of cells exposed to infection. Infection was not associated with morphological cell change, and growth was confined to cells infected at the time of each transfer of infection and the progeny of these cells. The bacterium is a microaerophilic, cell dependent, curved or rod-shaped, gram-negative bacillus that multiplies freely in the enterocyte cytoplasm. Cell cultures containing the intracellular bacteria appear to be free of other microorganisms, including chlamydiae and viruses.

The proliferative enteropathies are a group of pathologically similar diseases that affect a variety of animal species but principally pigs, hamsters, and, to a lesser extent, ferrets and rabbits. Affected intestines show enterocyte proliferation and the presence of intracellular, often curved, bacteria free within the cytoplasm. *Campylobacter* spp. can be recovered from such lesions (4, 5), but there is no consistency in the organisms that can be isolated from lesions in different species of animals. Most experimental infections with *Campylobacter* spp. have failed to initiate the disease or cell parasitism (1, 8). Certain pathological manifestations of the disease in pigs yield few *Campylobacter* organisms on culture (7) yet demonstrate numerous intracellular bacteria either by silver staining or electron microscopy; pathologically, such cases feature intestinal hemorrhage and are described as proliferative hemorrhagic enteropathy (PHE).

The intracellular organisms (IO) derived from such PHE cases are morphologically similar but antigenically distinct (9, 10) and by DNA analysis (11) are different from known *Campylobacter* spp. Unlike *Campylobacter* spp., no flagella have ever been noted on these IO. Also, intracellular bacteria in proliferative lesions of the hamster, ferret, and rabbit share a common antigen with IO from pig tissue (3, 6, 16).

An explanation for these observations is that the proliferative enteropathies are associated with a group of related organisms that have not as yet been cultivated in vitro. Others have proposed similar explanations, and Stills (17) claimed to have reproduced the disease in hamsters with pure cultures of organisms grown in cell culture. However, a later report (18) indicates that his cultures contained a variety of bacteria including chlamydiae. This report describes the cultivation in cells of a single morphologically distinct IO derived from pig tissues. Attempted reproduction of proliferative enteropathy with these cultured IO has not proved straightforward, and these results will be reported elsewhere.

MATERIALS AND METHODS

Source material. Material was obtained from the intestines of pigs with naturally occurring cases of histologically con-

firmed PHE (15). Fresh or frozen (-70°C) tissues were processed; the intestine was opened, the mucosa was removed and homogenized, an equal volume of 1% trypsin (Difco) in phosphate-buffered saline (0.1 M, pH 7.4) was added, and the suspension was incubated for 35 min at 37°C . Forty milliliters of Dulbecco's modified Eagle medium (DMEM; GIBCO no. 074.021.00) with 10% fetal calf serum (FCS) was added to 10 ml of trypsinized mucosa; the diluted tissue was ground in a tissue grinder and filtered through a 200-mesh stainless steel filter, through a glass fiber filter (Whatman GF/D), and through 1.2-, 0.8-, and 0.65- μm -pore-size membrane filters (Millipore, Watford, United Kingdom). Dimethyl sulfoxide was added to a final volume of 10% at room temperature, and aliquots were frozen at -70°C . Some filtrates were prepared by using similar techniques but using a sucrose potassium glutamate solution with 10% FCS as the diluent (2). The number of *Campylobacter* organisms, generally *C. coli* or *C. jejuni*, did not normally exceed $3.0 \log_{10} \text{g}^{-1}$ in the tissue of origin.

Cell culture. Rat small intestinal cells (IEC-18; ATCC CRL 1589) were grown in DMEM with 10% FCS, and the cells were trypsinized at weekly intervals and seeded at $0.5 \times 10^5 \text{ ml}^{-1}$ into fresh containers. Uninfected cells were grown in air with 5% CO_2 at 37°C in media without antibiotics but with L-glutamine (2 mM) and amphotericin B (2.0 $\mu\text{g/ml}$). IEC-18 appeared to be superior to the other cell lines tested, but a number of these (human fetal intestine, INT-407; rat colonic adenocarcinoma, CRL 1677; and pig kidney, PK-15) supported the growth of the organism. Others, including pig intestinal lines, proved unsuitable for a variety of reasons.

Infection of cell culture. IEC-18 cells were seeded onto 13- or 16-mm-diameter glass coverslips in 0.5- or 2.0-ml volumes of media either in bijou or universal-type containers; tissue culture flasks (catalog no. 690 175; Greiner) were seeded at comparable rates. Monolayers were exposed to individual source material filtrates at a variety of times after seeding. Frozen aliquots of the filtrates were thawed rapidly at 37°C , diluted in DMEM–7% FCS at ratios between 1:10 and 1:30, and added to the monolayer. Monolayer cultures were centrifuged at $2,020 \times g$ before transfer to anaerobic jars. Jars were evacuated by vacuum pump, and the gas was replaced with hydrogen and carbon dioxide to give a gas

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mixture of 8.0% O₂ and 7.0% CO₂. The cultures were incubated at 37°C for 3 h, refed if required, and then transferred to an incubator containing an atmosphere of 8.8% CO₂ and 8.0% O₂.

Neomycin or gentamicin (50 µg/liter) and vancomycin (100 µg/ml) were added to cultures exposed to infection. Infected cultures were refed every 2 to 3 days with DMEM-5% FCS with antibiotics.

Passage of infection. Passage of the infection was attempted by using the supernatant fluid of cultures or cell lysis and reinfection of fresh monolayers or by passage of trypsinized infected cells. The use of supernatant fluid or passage of trypsinized cells involved the standard techniques described; cell lysis involved exposure of infected monolayers to 0.2% KCl for 5 min and then to 0.1% KCl for 20 to 30 min at 37°C. Cells exposed to hypotonic KCl were drained, DMEM-7% FCS was added, the monolayer was detached mechanically from the flask, and the cells were lysed by passing the suspension repeatedly (six times) through a syringe with a 1½-in. 19-gauge needle. This procedure lysed the cells but left the nuclear membrane and nuclei intact. Cell nuclei were removed before fresh cells were infected by centrifugation at 100 × g for 5 min.

Monitoring of infection. The development of infection was monitored by phase microscopy of infected monolayers (Leitz Diavert; magnification, up to ×400) and the preparation of cytospin preparations from the supernatant fluids and trypsinized cells. Cytospins were sedimented at 750 × g for 10 min (Shandon Scientific Ltd., Runcorn, United Kingdom) to minimize cell damage. Coverslip preparations were harvested at appropriate intervals, washed in Locke's salt solution (12) for 5 min at 37°C, and fixed in acetone for 1 min or in methanol for 5 min before being stained. Staining was carried out either by the modified Ziehl-Neelsen technique or by immunofluorescence or immunoperoxidase methods. Both immunotechniques employed mouse monoclonal antibody IG4 (10) as the primary antibody (ascites) and either affinity-purified anti-mouse immunoglobulin G-fluorochrome conjugate (fluorescein isothiocyanate; Sigma) or peroxidase conjugate (sheep anti-mouse immunoglobulin G; Scottish Antibody Production Unit). Bacterial proliferation was assessed by counting the numbers of specifically stained bacteria (SIO) either associated with cells or in a unit area of the coverslip; further quantitation of infection is detailed in Results.

Presence of other bacteria. Monolayers exposed to infection were monitored by phase microscopy and cultivated onto bacterial growth media. This involved either sampling the supernatant fluid or mechanically removing the cell monolayer by swabbing. Cultivation was carried out in brucella semi-solid broth (catalog no. 0495-02-0; Difco) and Columbia blood agar incubated under microaerobic conditions at 37°C for up to 5 days and in reinforced clostridial media (CM149; Oxoid).

Selected infected coverslips were stained for the presence of chlamydiae by using Giemsa staining, anti-*C. trachomatis* outer membrane protein monoclonal antibody (Microtrak; Syva) or anti-chlamydial lipopolysaccharide (Imogen; Dako). Other coverslips were also stained indirectly by employing specific *C. psittaci* monoclonal antibody and monoclonal anti-chlamydial lipopolysaccharide (both kindly supplied by G. Jones, Moredun Research Institute), all with appropriate positive and negative control specimens. Indirect staining was completed with affinity-purified anti-mouse immunoglobulin G-fluorescein isothiocyanate.

Transmission electron microscopy. Monolayers were also

grown on Thermanox coverslips (ICN Flow), exposed to infection, harvested at various times after infection, and fixed in glutaraldehyde (1%) in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4°C. Some were treated with 0.5% Triton X-100 in the same buffer for 30 min, washed in buffer, and then stained by anti-IO IG4 with immunoperoxidase or immunogold labelling (1-nm-diameter gold bead-labelled goat anti-mouse immunoglobulin G; BioCell Laboratories). Ultrathin sections were prepared from selected fields, mounted on grids, stained with uranyl acetate and lead citrate, and examined with a Philips EM400 electron microscope.

National Collection of Type Cultures. Two strains of cell cultivated bacteria (NCTC 12656 and 12657) have been deposited with the National Collection of Type Cultures.

RESULTS

Light microscopy of primary infection. Infected monolayers examined by phase microscopy showed little morphological change; cytopathic effects, syncytia, vacuoles, and inclusions, or rounding of cells were not seen in association with infection. Monolayers exposed to infection incubated under reduced oxygen tension (8.0% O₂) grew at rates similar to those for cells grown in atmospheric oxygen, and detachment of confluent monolayers, although irregular, under either atmosphere took place after similar periods of incubation.

Occasional primary infections resulted in obvious contamination by rapidly growing extracellular bacteria, particularly coliforms, that were visible by phase microscopy; such contamination tended to be a feature of certain intestinal filtrates and could be overcome by refiltering (0.65-µm-pore-size filter) thawed aliquots immediately before cell infection. Other than these contaminating organisms, neither free nor intracellular bacteria could be identified by phase microscopy.

Immunostaining of IO. Either immunofluorescence or immunoperoxidase-stained preparations demonstrated large numbers of SIO with rod-shaped, S-shaped, or curved morphology, associated with cells after exposure to infection and centrifugation; noncentrifuged cells showed fewer bacteria; uninfected cells showed no staining. Immediately after infection, many cells (1 to 100% depending on the filtrate and dilution) had SIO associated with them. The distribution of bacteria between cells was not uniform and differed from that which might be expected in a Poisson distribution (P , <0.001). Some cells had no bacteria, but some cells often demonstrated 10 to 20 SIO closely associated with the cells, although it was rare for cells to have more than 30 SIO associated with a single cell at or during 2 days postinfection (p.i.), irrespective of the source or dilution of the intestinal filtrate. Some cells with large numbers of bacteria rounded off and appeared to be detaching from the monolayer during the first 48 h p.i.

During the first 2 days p.i., both the mean number of SIO cell⁻¹ and SIO mm⁻² decreased in monolayers exposed to infection and incubated in either 5% CO₂ or 8.5% CO₂ and 8.0% O₂. By 2 days p.i. in monolayers incubated under reduced oxygen, a few cells showed increasing numbers of SIO. As infected cells rarely demonstrated more than 30 SIO immediately after exposure, this level of infection was chosen for monitoring the proliferation of SIO, and such cells are identified as heavily infected cells (HIC). The numbers of HIC reached a maximum between 5 and 7 days p.i.; such cells contained organisms scattered throughout the

FIG. 1. IEC-18 cells infected with IO derived from cases of proliferative enteropathy were immunoperoxidase stained by employing anti-IO monoclonal antibodies and hematoxylin counterstain. (A) Isolated HIC. The cell cytoplasm is full of SIO. Magnification, $\times 576$. (B) HIC, with many surrounding cells also infected. The HIC contains many hundreds of bacteria. Magnification, $\times 720$. (C) Infected cells with fewer organisms. Some bacteria appear bloated, and other aggregates of antigen are circular and condensed (arrow). Magnification, $\times 1440$. (D) Groups of HIC. Many cells contain large numbers of bacteria (arrows), and some are detaching from the monolayer. In other adjacent cells, bacteria are less numerous. N, nuclei of HIC. Magnification, $\times 720$.

cytoplasm, and, in the most heavily infected cells, bacteria packed the cytoplasm but rarely extended into the supranuclear area (Fig. 1A and B). The majority of SIO were typically curved or rod-shaped bacteria, but some cultures contained aggregates of antigen from 3 to 6 μm in diameter (Fig. 1C). Organisms that had a spiral filamentous morphology but reacted typically with immunostaining were occasionally present.

HIC were frequently present as groups of cells, with adjacent cells that were also infected but contained fewer than 30 bacteria. Infected cells arranged in this way may have arisen from one or two infected cells, a view supported by the presence of infected cells undergoing cell division. Such dividing cells were either lightly or heavily infected. Groups of adjacent HIC were recorded as infected foci (Fig. 1D); infected foci were taken as a measure of the infectivity of the inoculum, while the number of HIC after incubation was taken as a measure of bacterial multiplication in the

monolayer. Infected foci might contain as many as 25 HIC. HIC were never observed in cells exposed to infection that were incubated under 5% CO_2 and oxygen at atmospheric tension (Table 1).

The number of HIC reached a peak at or about 5 days p.i. and thereafter remained relatively static or tended to decline. Most comparisons of the effects of treatments were made by harvesting coverslips at 5 or 6 days p.i.

Only a small number of cells (3 to 6%) in the monolayer had SIO associated with them by 5 to 7 days p.i., and the other cells remained healthy and uninfected. Neither Giemsa staining nor hematoxylin counterstaining of monolayers demonstrated bacteria, including any with *Campylobacter* morphology, nor was Giemsa stain capable of demonstrating HIC. Modified Ziehl-Neelsen stain enabled the detection of only a small proportion (10% or less) of the HIC detected by immunological methods.

Centrifugation of cells at infection was not essential for the

FIG. 1—Continued.

establishment of infection but enhanced the number of HIC in both primary and passaged infection. If cells were exposed to infection in antibiotic-free media and refed after 3 h, centrifugation was essential to obtain reasonable levels of infection (Table 2).

TABLE 1. Infection in IEC-18 cells exposed to strain 916/91 IO (derived from proliferative enteropathy intestine) by immunoperoxidase staining

Day p.i.	Infected cells (%) in ^a :		Number of HIC in ^b :	
	8.0% O ₂ + 8.8% CO ₂	5% CO ₂ in air	8.0% O ₂ + 8.8% CO ₂	5% CO ₂ in air
0	0.96	0.96	0	0
1	0	0.32	0	0
2	0.81	0.32	0	0
5	6.29	0.32	380	0
7	2.90	0.12	133	0

^a Percentage of cells infected calculated for three to six coverslips, with 200 cells counted on each.

^b Mean total number for three to six coverslips.

Isolation from clinical material. Multiplication of IO, as assessed by the appearance of HIC, was established in 83% of the experiments ($n = 101$) using tissues derived from 10 PHE cases originating from four separate farms. All tissues yielded SIO in culture on at least one occasion. Although considerable differences existed in the infectivity of different filtrates, SIO was recovered from both freshly prepared frozen aliquots and tissues that had been frozen and thawed before preparation and storage. The main reason identified for culture failure (6%) was the growth of contaminating bacteria, particularly coliforms.

Effect of trypsin and antibiotics on infection. Freshly trypsinized cells were more susceptible to infection than day-old monolayers adjusted to similar cell concentrations (Table 2). Infection was established in the presence of neomycin, but the omission of neomycin during the 3-h p.i. period resulted in a significant enhancement of infection from a mean of 84 to 236 HIC that developed 5 and 6 days after infection (Table 2); gentamicin affected infection in a manner similar to neomycin.

Passage of infection. Passage of infection was achieved either by use of lysis, trypsinization, or the supernatant fluid

TABLE 2. Appearance of HIC in monolayers of IEC-18 cells infected with strain 1378/90 of IO under various conditions

Characteristic	Mean no. of HIC ^a
Centrifugation	
With.....	30.5 (2-47)
Without	7.5 (0-30)
Monolayer age ^b	
0.....	49.5 (16-93)
1 day	30.5 (2-47)
Presence of neomycin	
At infection	84 (5-256) ^c
3 h p.i.....	236 (48-665) ^c

^a The mean number of HIC that developed for four coverslips harvested at day 5 to 6 p.i. The range of HIC is given in parentheses.

^b Monolayers with similar cell concentrations at infection; 0, freshly trypsinized cells; 1, day-old monolayer.

^c Difference was significant ($P < 0.05$).

from infected monolayers. Of the three methods, cell lysis proved the most useful in maintaining infection, and it was difficult to retain infectivity by either of the latter two methods. Infection has been maintained for 20 passages by using cell lysis for transfer.

HIC appeared sooner in monolayers exposed to supernatants or cell lysates from heavily infected cell cultures (Fig. 2, day 2), increased to a maximum at day 7, and then declined slowly over the remaining period of the experiment to day 11. Phase microscopy showed cells free in the supernatant fluid often appearing at a maximum at day 5. Stained cytopspins of such cells showed some to contain IO; noninfected cultures showed similar, but fewer, extruded cells by phase microscopy.

Immunostaining of monolayers in the first 2 days p.i. showed that the proportion of infected cells declined from 17.2 to 7.5%, and despite the large numbers of cells with bacteria closely associated at this time, only a minority of these organisms later multiplied to produce HIC. This pattern was a feature not only of infective cell lysates but also of supernatants; similar results were also obtained with another cell lysing agent, octylglucopyranoside (data not presented).

The rate of cell division in infected monolayers was similar to that seen in noninfected monolayers cultivated under reduced oxygen tension. The cell-seeding density affected the number and rate of appearance of HIC. Bacterial proliferation appeared to be maximal with cell-seeding densities of 0.25×10^5 to 0.5×10^5 cells ml⁻¹, and lower and higher cell numbers yielded fewer HIC; low cell densities and low inoculum infectivity could result in an absence of bacterial proliferation. Other features of passage were similar to those of primary infection.

Although passage of trypsinized infected cells was readily achieved, it was difficult to consistently maintain a high number of HIC. Trypsinization of monolayers infected early (day 1 p.i.) and late (day 11 p.i.) failed to result in the transfer of infection; monolayers trypsinized between these periods yielded monolayers containing HIC. In monolayers set up by trypsinization of heavily infected monolayers, the number of infected cells and HIC progressively expanded during the days following seeding but often approached only a third of the number present in the seeding culture by day 7 p.i. A comparison of cytopspins of trypsinized cells before seed-

FIG. 2. IEC-18 cells exposed to infected supernatant fluid from IEC-18 cells infected with IO of strain 916/91 (passage 12). Infected cells were assessed by counting 200 cells; the HIC density was calculated from the total number of HIC; cell density was derived from nuclear counts in five areas of 0.0672 mm^2 . All results are the mean values for three coverslips.

ing and those adherent after 30 min indicated that fewer than 1 in 200 HIC adhered to the surfaces being seeded in this period.

Examinations for other bacteria. Neither cultures from supernatant fluid nor cell scrapes from infected cultures yielded bacteria when grown on cell-free media. Monolayers from repeatedly passaged cultures of two strains of IO failed to show any evidence either of chlamydial elementary bodies or inclusions by either Giemsa staining or immunostaining.

Electron microscopy. IO-infected cells showed numerous bacterial forms free in the cell cytoplasm and not bound by a cell membrane (Fig. 3, inset). The outer membrane of such bacteria was specifically stained in sections stained with IG4 and immunogold labelling. Bacteria were $0.3 \mu\text{m}$ in diameter and $1.0 \mu\text{m}$ in length, curved or rod shaped, with a trilaminar outer membrane often separated from the cytoplasmic membrane by an electronlucent zone (Fig. 3). Inclusions contain-

FIG. 3. Ultrastructure of IEC-18 cells infected with IO derived from cases of proliferative enteropathy at day 6 p.i. The cells contain numerous IO. Bacteria lie freely within cell cytoplasm, with no evidence of a cell membrane, as shown by uranyl acetate-lead citrate staining. Magnification, $\times 77,000$. Bar, $0.1 \mu\text{m}$. (Inset) A group of irregularly curved bacteria lie freely in the cytoplasm, as shown by uranyl acetate-lead citrate staining. Magnification, $\times 6,000$.

ing numerous bacterial forms enclosed within a membrane-bound structure were not detected.

DISCUSSION

Research on the proliferative enteropathies has been seriously hampered by (i) confusion over the identity of and (ii) the inability to cultivate the etiological agent. Previous reports have identified the location, morphology, antigenicity, and specific genetic features (9, 11, 14) of the intracellular bacteria; therefore, the identification of any cultivated microorganism must satisfy these criteria. This study confirms that an intracellular bacterium that cannot yet be

cultivated on conventional media can be regularly cultivated from PHE cases in enterocytes of rat origin, that the organism is located free within the cytoplasm of the cultured cells, is morphologically compatible with IO, and is identifiable in both light and ultrastructure preparations by immunological means previously shown to be specific for the IO that is associated with the field disease. The occasional presence of specifically staining aggregates of antigen and bacterial spiral filaments probably merely reflects suboptimal growth of the IO. The immunostaining of cells exposed to infection provides strong evidence for the multiplication of the bacteria; organisms are localized to groups of cells within the monolayer, and infection does not spread to

adjacent cells once the monolayer has reached maturity. Antibiotics have had to be used to suppress contaminating bacteria, and neomycin has been shown to modify the infection stage of IO. Such antibiotics might be expected to depress extracellular multiplication of IO; however, removal of neomycin from passaged cultures did not increase the number of HIC (data not presented). This indicates both that little transfer of infection takes place to uninfected cells in the monolayer during the course of infection and that multiplication of IO is highly cell dependent.

Intracellular growth of the bacteria produces little morphological cell alteration and no lysis, both features of the naturally occurring disease. All these results indicate that the organism that we have cultivated is the one that can be visualized in tissue with proliferative enteropathic disease.

These results also provide additional evidence of the unique characteristics of the bacteria that are associated with proliferative enteropathy. The bacteria isolated are obligate, intracellular, gram-negative curved rods which tolerate oxygen only at reduced pressure from atmospheric tension and grow in enterocytes only in vivo, characteristics which are distinctive among the described bacterial pathogens of mammals. Two strains (NCTC 12656 and NCTC 12657) of the bacteria have been deposited with the National Collection of Type Cultures. The morphological and quantitative data on cell infection would support the view that much of the spread of infection within infected monolayers takes place by division of infected cells, with little spread beyond those cells infected during the initial exposure. Although supernatant fluids contain viable infectious bacteria, there is little evidence for late transfer of infection to previously uninfected cells in the culture. These features possess some of the characteristics postulated for the pathogenesis of the naturally occurring disease in which crypt cells are initially infected, with subsequent spread of the bacteria within dividing cells to the surrounding epithelium.

The cell culture system described has many deficiencies, not the least of which is the apparent failure of many bacteria to establish cell infection once associated with cells. It does, however, provide a basis for regularly cultivating these organisms from infected pig tissues, which will allow more detailed examination of the microbe and the disease with which it is associated. In addition, it may provide a laboratory model for investigation of some aspects of the naturally occurring disease.

The failure to isolate conventional bacteria or demonstrate chlamydiae indicates that the intracellular agent may be present in pure culture, although the presence of noncytopathic viruses remains relatively unexplored. Others have reported a more confused pattern and the presence of atypical chlamydiae in cell culture, possibly along with IO (18).

Chlamydia are common parasites of the intestinal tract of neonates (13) of a variety of animal species. They are generally considered to have little pathological significance. Our choice of using PHE cases for isolation was made because of the small numbers of cultivable *Campylobacter* spp. in the intestinal tissues of infected animals; such animals are mature and so also may prove less likely to be contaminated by *Chlamydia* spp.

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