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Reproduction of Porcine Proliferative Enteropathy with Pure Cultures of Ileal Symbiont Intracellularis

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Porcine proliferative enteropathy is consistently associated with the presence of intracellular curved bacteria in epithelial cells in affected portions of intestine. Two strains of these intracellular bacteria were cultured in a cell culture system with rat enterocytes (IEC-18) and passaged several times and used as oral inocula for 14 gnotobiotic and 8 conventional pigs. DNA and immunological studies had identified these bacteria as belonging to a new taxon, Ileal symbiont (IS) intracellularis. Conventional pigs dosed with approximately 3.7×10^6 of these organisms passaged six times in cell culture developed severe lesions of proliferative enteropathy in the ileum. Other conventional pigs dosed with a lower titer or with organisms passaged 13 times developed moderate and minor lesions, respectively. All gnotobiotic pigs dosed with organisms failed to develop lesions. Control pigs, eight conventional and two gnotobiotic, dosed with diluent, uninfected cell material or left undosed failed to develop lesions also. Reisolation of IS intracellularis and demonstration of the organism in mucosal and fecal samples only occurred in conventional pigs dosed with organisms. Gnotobiotic pigs lacking a normal intestinal flora have not been shown to be colonized by the organism. Seroconversion to IS intracellularis or mucosal infiltration by inflammatory cells was not observed in experimentally affected pigs, confirming the weak immune response characteristic of the natural disease. These results support the identification of IS intracellularis as an etiological agent of proliferative enteropathy in pigs.

Proliferative enteropathy in pigs was first recognized as a specific disease in the United States in 1931 (1), with characteristic lesions of marked adenomatous hyperplasia of the intestinal mucosa in the ileum and colon. Subsequently, curved gram-negative bacilli within the cytoplasm of proliferative epithelial cells of the intestinal crypts within affected regions of bowel were found to be a consistent finding (23). Clear evidence of transmission of the disease between pigs has been demonstrated only when homogenates of affected mucosae containing numerous intracellular bacteria were used as oral inocula (13, 22). Culture of various curved bacteria, particularly *Campylobacter* species, from the lesions or "successful" inocula onto standard bacteriologic media has occurred (8, 11). However, use of these bacteria as oral inocula has never reproduced the specific disease (2, 14). The conclusion that the intracellular bacteria in the lesions were likely to belong to a separate group of *Campylobacter*-like organisms requiring cells for growth was supported by immunologic and DNA studies on intracellular bacteria purified from the lesions (6, 15). This led to our recent development of a successful cell culture system for the intracellular bacteria, using the rat enterocytes IEC-18 (10).

Further DNA analysis of purified and cultured intracellular bacteria established that they belong to a new taxon within the delta group of the proteobacteria for which the name Ileal symbiont (IS) intracellularis is currently proposed (5, 18), although further taxonomic study is in progress. The clear identity of IS intracellularis with the intracellular bacteria in the lesions was established by immunostaining with specific monoclonal antibodies (16) and by in situ hybridization with specific DNA probes (7). However, there is a possibility that other agents, such as viruses or *Chlamydia* species, are involved in some primary role in the disease with subsequent cell entry of bacteria (13, 25). To examine the ability of IS intracellularis alone to cause proliferative enteropathy, we used organisms grown in pure culture in enterocytes as oral inocula for pigs.

MATERIALS AND METHODS

Gnotobiotic pigs. Sixteen pigs were delivered by cesarean section under sterile conditions and maintained in sterile plastic isolators as described previously (17). Pigs were fed evaporated cow's milk supplemented with vitamins and minerals and dosed orally at 7 days of age. Monitoring of pigs by aerobic and anaerobic bacterial culture of fecal swabs at 1 and 7 days of age revealed *Streptococcus* sp. in two pigs, but no other isolates.

Conventional pigs. Sixteen pigs were delivered from specific-pathogen-free sows and maintained in sanitary conditions separate from other pigs. The herd had never had a recorded case of proliferative enteropathy in its 2-year history. Piglets were weaned from the sows at 28 days, without the use of any supplementary creep feed, into separate sanitary pens for dosed and control groups. Pigs were dosed orally at 29 days of age and fed ad libitum commercial pig feed free of any antibiotics.

Pig-derived bacteria. The source material used for infection of cells was derived from the affected intestines of two pigs naturally affected with proliferative hemorrhagic enteropathy designated 916/91 and 1482/89. The method of preparation of pure suspensions of intracellular bacteria from the lesions has been described previously (16). These bacterial suspensions were suspended finally in sucrose-potassium-

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glutamate (3) containing 10% (vol/vol) fetal calf serum or in tissue culture growth media with 10% (vol/vol) dimethyl sulfoxide in 1-ml vials. Prepared vials were stored frozen at −70°C for 3 to 4 months, respectively, before they were used.

Tissue culture-derived inocula for pig infections. IEC-18 cells, rat enterocytes (American Type Culture Collection no. CRL 1589), were grown to a monolayer covering 20 to 30% of 25-cm^2 plastic flasks in 24 h in Dulbecco's modified Eagle's medium supplemented with L-glutamine, amphotericin B (Fungizone), and 10% (vol/vol) fetal calf serum. Infection of these cells by intracellular bacteria, IS intracellularis, derived from pig intestines is described in detail elsewhere (10). That study had cultured isolates from 10 pigs from four farms; two strains, 1482/89 and 916/91, from different farms were selected for this study. These two strains had been deposited previously in the National Collection of Type Cultures, London, England, as NCTC 12656 and 12657, respectively (10).

Briefly, after rapidly thawing at 37°C, one 1-ml vial of pig-derived bacteria was added to 14 ml of warm Dulbecco's modified Eagle's medium with supplements as given above but with 7% (vol/vol) fetal calf serum. The diluted suspension was then added to the IEC-18 cell monolayers in two 25-cm^2 flasks. Infection of cells was assisted by centrifugation of the flasks at 2,000 × *g* for 30 min. Flasks were incubated microaerobically (8% O_2, 8.8% CO_2, 82% N_2) for 3 h. The cells were then fed with the same medium containing neomycin and vancomycin and further incubated as described above. At days 2 and 4 postinfection, the infected cell monolayers were further refed with the same growth medium, supplements, and antibiotics, but with 5% (vol/vol) fetal calf serum, and finally harvested at day 6 postinoculation for passage.

Passaging of infected cells was performed by treatment with potassium chloride, as described by Lawson et al. (10), and then removal of the cells in each flask with a cell scraper. The scraped cells were then ruptured by passage six times through a needle before they were used to infect fresh monolayers of IEC-18 cells in new 25- or 75-cm^2 flasks.

The infected new cells were grown for another 5 to 7 days before they were prepared for either infecting pigs or repassaging. The number of passages of bacteria grown in cells and used to infect each pig is given in Table 1. For each flask, the medium was removed and replaced with 2 ml of growth medium or sucrose-potassium-glutamate with 5% (vol/vol) fetal calf serum, and the cells were removed with a cell scraper. Cells from each passage used for each group were then bulked and homogenized briefly for 15 s, and pigs were dosed orally with 10 ml each via a syringe and tube. Introduction of inocula into the isolator for gnotobiotic pigs entailed a delay of 1 to 2 h after homogenization before dosage of pigs.

Control inocula. Control pigs were either not dosed or dosed orally with 10 ml of sucrose-potassium-glutamate or with infected IEC-18 cells which had been passed through a 0.22-μm filter (Table 1).

Monitoring of inocula. To monitor the numbers of IS intracellularis in each IEC-18 passage, in addition to the infected flasks, parallel infections of IEC-18 cells on glass coverslips cultured in identical media in small vials were conducted. On day 5 or 7 postinfection, the relevant coverslips were collected at the same time as the infected flasks, washed in warm Locke's salt solution (21), fixed in acetone, and mounted on glass slides for specific immunoperoxidase staining. Coverslips were stained with monoclonal antibody IG4 as the primary antibody and an anti-mouse peroxidase conjugate used as the secondary antibody in an indirect immunoperoxidase test with hematoxylin counterstaining. Antibody IG4 is known to be specific for IS intracellularis (16).

In addition, 10 μl of each bulked inoculum used to dose pigs was smeared onto a glass slide, and the slides were air dried, fixed in acetone, and stained by an indirect immunofluorescence assay, using the same primary antibody IG4 and then fluorescein-conjugated sheep anti-mouse antibody. Infected and noninfected IEC-18 cells stained for the presence of *Chlamydia* species, using Giemsa staining and either anti-*Chlamydia* lipopolysaccharide or membrane protein antibody kits obtained commercially, were uniformly negative.

Necropsy. All pigs were euthanized 22 days after dosing, and a full necropsy was performed. Samples of stomach, duodenum, jejunum, ileum (three portions), cecum, proximal and spiral colon, and ileocecal and mesenteric lymph nodes were immersed in 10% buffered formalin for fixation. Sections for light microscopy examination were stained by routine hematoxylin and eosin and Young's silver stain (17). Samples of jejunum, ileum (three portions), cecum, and proximal colon were immersed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for fixation. Sections for transmission electron microscopy examination were prepared as described previously (17). Separate sections of each sample were stained in an indirect immunofluorescence

TABLE 1. Inocula

Pig no.	IS intracellularis strain	No. of passages in IEC-18 cells	Inoculum smear[a]	Inoculum monitor (no. of HIC/no. of foci of infection)[b]
Gnotobiotic				
1	916/91	3	+/−	0/0
2		5	+	3/2
3		6	+/−	0/0
4		13	+	2/1
5		13[c]	++	60/19
6		13[d]	0	
7	1482/89	11	+/−	0/0
8		13	+	8/2
9		13[d]	0	
10		14	+	1/1
11		16	+	16/1
12		19[c]	+++	284/53
13, 14		20	+++	318/158
15, 16		21	+++	650/200
Conventional				
1, 2	916/91	6	+++	3590/164
3, 4		6	+++	1388/282
5, 6, 7, 8		13	+++	219/49
9, 10	SPG[e]		0	
11, 12, 13	Not dosed			
14, 15, 16	Not dosed			

[a] Ten microliters of inoculum was immunostained, and smears were examined for IS intracellularis. +++, >100 bacteria per high-power field; ++, 10 to 100; +, 1 to 10; +/−, occasional; 0, none seen.

[b] Three coverslips were infected in parallel to inoculum flasks, immunostained, and examined for IS intracellularis. HIC, heavily infected cells. The values shown are means for three coverslips, with each coverslip = 130 mm^2. Heavily infected cells contain >30 organisms.

[c] Inocula were stored frozen at −70°C for 3 months prior to dosing.

[d] Inoculum was filtered through a 0.22-μm-pore-size filter prior to dosing.

[e] SPG, sucrose potassium glutamate diluent.

assay, incorporating specific monoclonal antibody IG4 for IS intracellularis, as described previously (16).

Serology and culture. Pigs were bled, and fecal swabs were collected at dosing and just prior to necropsy. Each serum sample was collected following centrifugation and incorporated into an indirect immunofluorescence assay using IS intracellularis whole-cell antigen 1269/76 to detect specific immunoglobulin M titers, as described previously (9). Intestinal swabs were cultured for *Campylobacter* species by routine methods.

Fresh samples of ileum were collected at necropsy, and portions of the mucosa were scraped into sucrose-potassium-glutamate diluent. Smears of each mucosa were prepared on glass slides and stained with a modified Ziehl-Neelsen stain, as described previously (12). Dilutions of each pig ileum mucosa were prepared for inoculation onto rat enterocyte cultures, as described above. Assessment of these inoculated rat enterocyte cultures for IS intracellularis infection was performed by immunostaining, as described previously (10).

Comparison of IS intracellularis isolates. Isolates of IS intracellularis obtained from the initial source material 916/91 and 1482/89, the inocula used, and from experimental pigs at necropsy were used to prepare bacterial DNA for comparison of each isolate. Specific DNA primers for IS intracellularis had been constructed from known sequences of DNA in the 16S rDNA region of its genome by Gebhart et al. (5). DNA prepared from each isolate was reacted with these primers, which were kindly supplied by C. J. Gebhart, University of Minnesota, under standard polymerase chain reaction (PCR) conditions.

RESULTS

Inocula. An outline of the inocula used in gnotobiotic and conventional pigs is given in Table 1. Bacterial culture of the tissue culture-derived inocula on cell-free media revealed no detectable isolates. Immunoperoxidase staining of infected IEC-18 cells set up in parallel to the flasks used to dose the pigs and smears of each inoculum examined under immunostaining showed few to numerous curved bacteria reactive with the primary monoclonal antibody (Table 1). Examination of infected monolayers showed no discernible cytopathic effects during infection and passage of bacteria. Examination of smears of filtered (0.22-μm pore size) inoculum showed no visible bacteria.

An estimate of the dose received by each conventional pig was derived by assuming that each heavily infected cell contained 50 organisms and that the infected cells were spread evenly over the flasks (75 cm^2) used for inocula and the monitoring coverslips (130 mm^2). With these assumptions, it is estimated that pigs 1 and 2 received no less than 3.7×10^6 organisms, pigs 3 and 4 received not less than 1.5×10^6 organisms, and pigs 5 to 8 received not less than 4.0×10^5 organisms.

All pigs remained clinically healthy throughout. Average weight gains of conventional pigs, both test and control, were similar, 0.39 and 0.42 kg/week, respectively. No diarrhea was observed.

Gross pathology. No gross lesions were detected in any gnotobiotic pigs or control conventional pigs at necropsy. In conventional pigs 2 and 3, mild thickening of the ileal and colonic mucosae was observed, particularly overlying mucosal Peyer's patches. No other gross lesions were detected.

Microscopic pathology. (i) Gnotobiotic pigs. No significant lesions were detected in any pig, with no significant infiltration of inflammatory cells into the lamina propria or any significant degree of crypt hyperplasia or proliferation. No intestinal bacteria were observed, inside or outside cells.

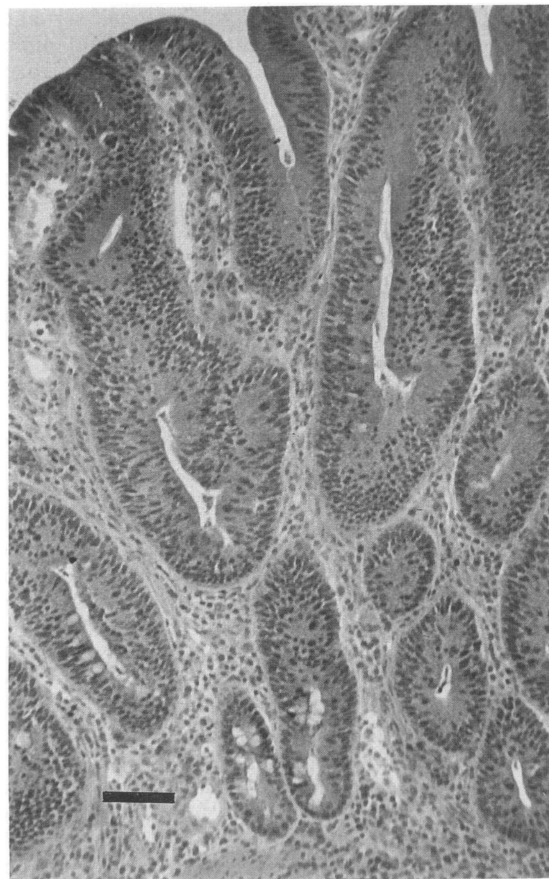

FIG. 1. Section of ileum from conventional pig 1. Note complete replacement of normal crypt/villus architecture by enlarged proliferative crypts. Section was stained with hematoxylin and eosin. Bar = 40 μm.

(ii) Conventional pigs. Diffuse microscopic lesions consistent with proliferative enteropathy were seen in all four conventional pigs, pigs 1 to 4, dosed with IS intracellularis 916/91 passaged six times. In pigs 1 and 2, which received a higher dose, there was almost complete replacement of normal ileal mucosa by adenomatous mucosa (Fig. 1). Affected crypts were enlarged and branched, with loss or absence of goblet cells and marked proliferation of crypt epithelial cells, with many mitoses apparent. There was some variation in the local severity of these lesions, which extended from the ileum to the colon in pigs 1 and 2, but these were somewhat more patchy in pigs 3 and 4 and were confined mostly to the terminal ileum (Table 2). In areas of ileum mucosa in pigs 3 and 4, clearly affected crypts could be found adjacent to normal crypts (Fig. 2). In pigs 5 to 8, only occasional microscopic lesions consistent with proliferative enteropathy were detected, with approximately one to four lesions in every 100 crypts in the terminal ileum being clearly affected in each pig. A summary of results, with ratios of crypt heights for each conventional pig, is given in Table 2.

The lamina propria of both affected and nonaffected intestines showed a mixed population of lymphocytes, eosinophils, and neutrophils. There was no apparent infiltra-

TABLE 2. Some results obtained in conventional pigs

Pig no.	% Histological lesions of proliferative enteropathy[a]					Crypt/villus ratio (mid-ileum)[b]	Examination for IS intracellularis[c]	
	Jejunum	Ileum	Cecum	Proximal colon	Spiral colon		Ileum mucosa cell culture (no. of HIC/no. of foci)	Fecal smear
1	0	99	1	1	0	0.85	6/2	+++
2	0	64	5	8	0	0.52	29/8	+++
3	0	8	0	1	0	0.41	16/6	ND
4	0.4	5	0	0	0	0.42	5/1	ND
5	0	1	0	0	0	0.38	0/0	ND
6	0	2	0	0	0	0.44		ND
7	0	4	0	0	0	0.35		ND
8	0	0.5	0	0	0	0.40		ND
9–16	0	0	0	0	0	0.36–0.42	0/0	ND

[a] Percentage of crypts that were enlarged and positive for intracellular bacteria of the total number of intestinal crypts in two silver-stained sections of each area.

[b] Obtained by dividing the crypt height by the total crypt and villus height for 50 crypt/villus units.

[c] Colonization of each pig by IS intracellularis was measured by culture of ileum mucosa preparation on IEC-18 cells as described in the text. Values given are the means for three coverslips (see Table 1, footnote *b*). A smear of each pig's feces was immunostained as described previously (16): +++, numerous; ND, no organisms detected.

tion of any of these or other cell types into mucosae with proliferative crypts. Microscopic examination of the lymphoid Peyer's patches and nodes in some conventional pigs showed moderate hyperplasia of follicular and interfollicular lymphocytes. No lesions were detected in other viscera. Silver stains showed numerous curved bacteria in the apical cytoplasm of epithelial cells within enlarged, affected crypts in the intestine (Fig. 3). Similarly, immunostaining of sections of affected intestine with specific monoclonal antibody IG4 showed numerous brightly fluorescing curved bacteria in the apical cytoplasm of epithelial cells in affected crypts. Some fluorescing bacteria were also detected in macrophages adjacent to affected crypts (Fig. 4). Curved bacteria stained by either method were not detected intra- or extracellularly in other parts of affected intestines, which appeared normal histologically in control pigs.

Electron microscopy. In conventional pigs, ultrastructural examination of sections of affected intestine showed marked proliferation of immature crypt enterocytes, containing numerous intracellular curved bacteria, free in the apical cytoplasm (Fig. 5). The bacteria were approximately 0.4 μm wide by 2.0 μm long and had a wavy trilaminar cell wall. Numerous bacterial divisions were evident. In some instances, the bacteria were very closely associated with cell mitochondria and rough endoplasmic reticulum, with some disruption of these adjacent organelles evident (Fig. 6). No such bacteria were identified in the lumen of any adjacent crypt in the ileum of affected pigs. No viruses or *Chlamydia*-like organisms were detected in any affected cells. No

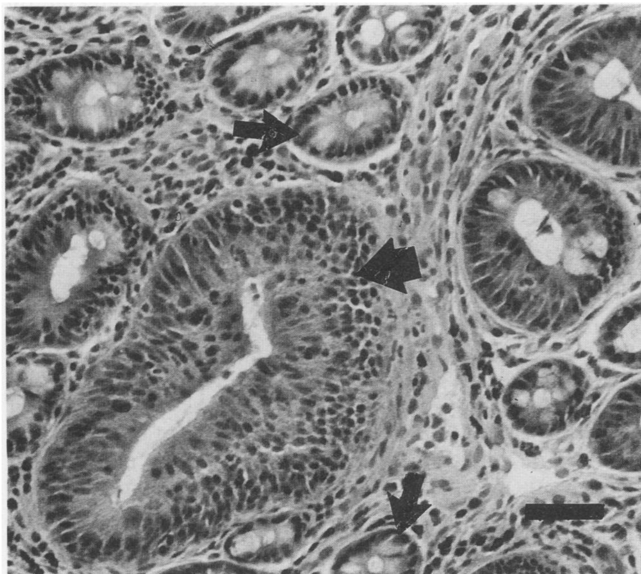

FIG. 2. Section of ileum from conventional pig 4. Individual affected crypts (large arrow) are thickened to 10 or more layers of immature epithelial cells compared with normal adjacent crypts (small arrows). Note absence of goblet cells in affected crypts. Section was stained with hematoxylin and eosin. Bar = 30 μm.

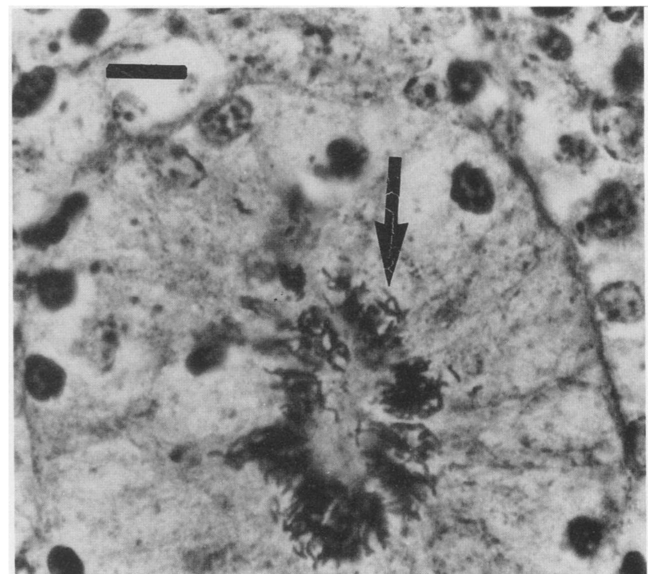

FIG. 3. Section of ileum from conventional pig 1. Note numerous curved intracellular bacteria (arrow) in the apical cytoplasm of proliferating epithelial cells. Section was stained with Young's silver stain. Bar = 10 μm.

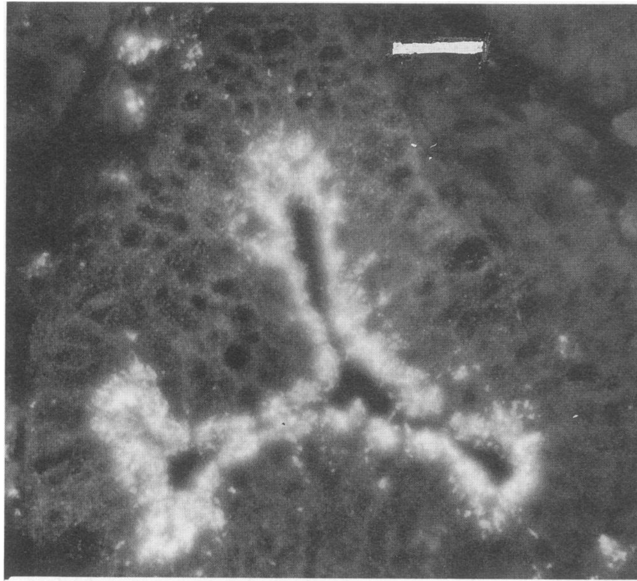

FIG. 4. Section of ileum from conventional pig 2. Note numerous brightly fluorescing curved bacteria in the apical cytoplasm of proliferating epithelial cells and macrophages in adjacent lamina propria. Immunostain for IS intracellularis was used. Bar = 20 μm.

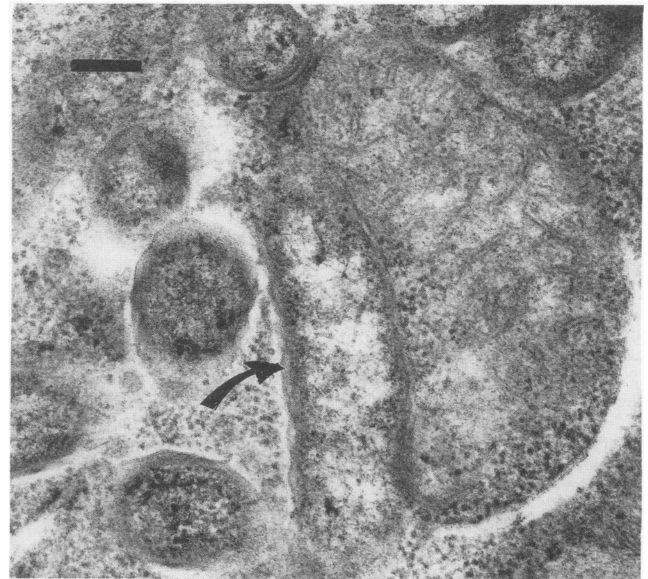

FIG. 6. Ultrathin section of ileum from conventional pig 1. Bacteria (arrow) are clearly associated with mitochondrium. Bar = 0.2 μm.

intracellular bacteria were detected in any control or gnotobiotic pig intestines.

Culture and serology and PCR testing. Immunostained fecal smears and mucosal smears stained by modified Ziehl-Neelsen stain were only clearly positive for acid-fast organisms in conventional pigs 1 and 2. *Campylobacter fetus* was cultivated from the ileum of conventional pigs 3, 4, 8, 9, and 10, and *Campylobacter coli* was cultivated from conventional pigs 12 and 14. IS intracellularis was recultured in rat enterocytes from the ileum mucosa of affected conventional pigs only (Table 2). PCR analysis of original 1482/89 and 916/91 intracellular bacteria, the 916/91 inocula, and strains recultured from conventional pigs 1, 2, 3, and 4 indicated that they shared identical gene sequences in the 16S rDNA portion of their genome, capable of reacting with primers specific for IS intracellularis. Reactions with each of these samples produced a PCR product of 319 bp, as detected by gel electrophoresis. None of the samples of any control pig intestines produced any recognizable PCR product with these primers. Intracellular bacteria were not cultured from any gnotobiotic pig intestines, despite several efforts.

No detectable titers were found in any pig serum. Control positive sera from natural cases of proliferative enteropathy were consistently positive at 1/360 dilution in this assay.

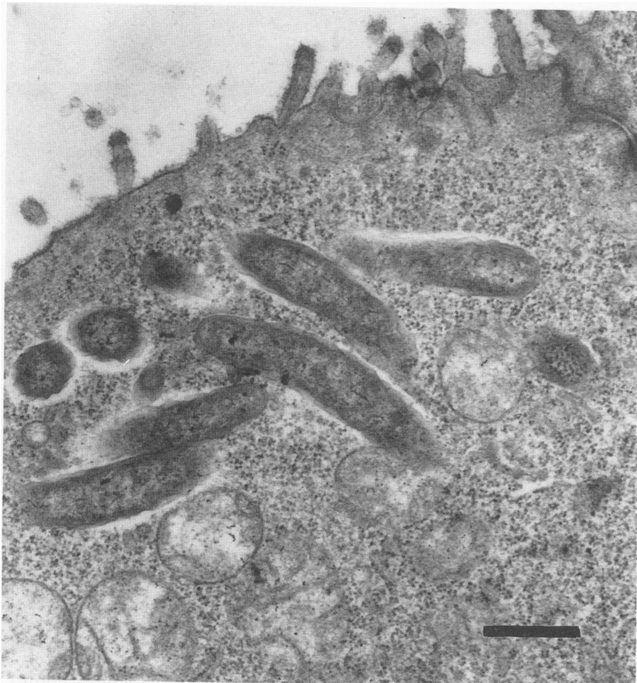

FIG. 5. Ultrathin section of ileum from conventional pig 1. Note curved intracytoplasmic bacteria in apical cytoplasm of immature epithelial cells. Bar = 1 μm.

DISCUSSION

This study is the first to report clear reproduction of proliferative enteropathy disease in pigs with a pure culture of an identifiable agent, IS intracellularis. Previous reports had indicated that this newly recognized organism was the intracellular bacterium present in lesions in the natural disease (7, 16), but this report establishes its etiologic role in the disease. Similar results have been reported in proliferative enteritis in hamsters (24), but that report did not identify the agent used, and a subsequent report indicated that their cell cultures were contaminated by a *Chlamydia* species (25).

IS intracellularis is an obligate intracellular bacterium which DNA analysis indicates is in the delta group of proteobacteria (5). However, it is clearly distinct from other known forms of bacterial life, and much interest surrounds both its relation to an important animal disease and the

unique nature of the bacterium-associated proliferative lesion.

It was clear from our study that IS intracellularis could only cause disease when conventional pigs were dosed, as no gnotobiotic pigs developed disease or even became colonized. This is similar to another pathogen of pigs, *Serpulina hyodysenteriae*, the causative agent of swine dysentery, which requires that an anaerobic flora, such as *Bacteroides vulgatas*, exist in the lower bowel before it can initiate colonic disease (27). However, one study showed that some strains of *S. hyodysentariae* could colonize the lower bowel of gnotobiotic pigs, although their pathogenicity was enhanced markedly by the addition of anaerobic flora (26). Further experiments may clarify whether this requirement is also important for IS intracellularis. It is also possible that the age or diet of gnotobiotic pigs modulates infection. However, previously we have reproduced proliferative enteropathy in gnotobiotic pigs dosed at 7 days of age with oral inocula consisting of IS intracellularis organisms partially purified from homogenates of affected intestines by crude filtration (17). It is also possible, therefore, that the organism somehow loses its pathogenicity for gnotobiotic pigs when cultured in vitro. More likely, a combination of these factors caused the failure of gnotobiotic pigs to develop disease or even support colonization of IS intracellularis in their intestines, in clear contrast to conventional pigs.

The major problem with the use of conventional pigs, one which has bedevilled proliferative enteropathy experiments in the past, is that the disease is very common; therefore, both spontaneous disease in control animals and resistance in test animals are clear possibilities. Great care was taken to minimize this problem by use of animals kept in separate sanitary pens from sows bred on a minimal disease farm with no evidence of proliferative enteropathy. All controls were clearly negative, and cultural isolation only occurred in dosed, affected pigs. Another result indicating that the response seen was due to our inocula was the clear dose- and passage-response differences seen in dosed pigs. Those pigs which received the highest dose of strain 916/91 passaged only six times developed severe pathologic lesions, whereas those pigs which received a smaller dose of the same organism developed moderate lesions and those which received the strain passaged 13 times developed minor lesions (Table 2), unlikely to have had any clinical impact.

The doses used were considered to be relatively small but were similar for groups receiving each passage, indicating that differences in lesions produced may be due to a diminution in pathogenicity of an isolate passaged multiply in vitro. Many species of bacteria exhibit a similar reduction in virulence on repeated subculture (20). It is possible that some of the machinery necessary for bacterial adherence, entry, and intracellular survival in vivo becomes redundant when cultures are grown in cell culture systems. We did not demonstrate any ability of strain 1482/89 to initiate lesions, although we did not use it in conventional pigs. Differences in the rates at which strains of IS intracellularis become less virulent in culture may occur. Conventional pigs given few intracellular bacteria only developed minor pathological changes. The presence of additional noncytopathic agents in infected cell culture cannot be excluded. The apparent close relationship between dose and pathological response, however, strengthens the argument that IS intracellularis is the primary organism.

Some features of the natural disease were clarified by these experimental studies. The location, extent, and timing of development of lesions were similar to those suggested previously for the natural disease and previous experiments which used mucosal homogenates as inocula (13, 17).

The weak immune response mounted in the host, reflected by our failure to detect specific immunoglobulin M titers, and the nonappearance of mucosal infiltrates of inflammatory cells in affected pigs are consistent with our previous studies (19), which suggested that IS intracellularis enjoys a considerable sheltering from host immunity by inhabiting enterocytes, which lack major histocompatibility complex class II, although its presence in some mucosal macrophages indicates that a host response will eventually occur in severe lesions, as a previous serological study indicated (9).

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